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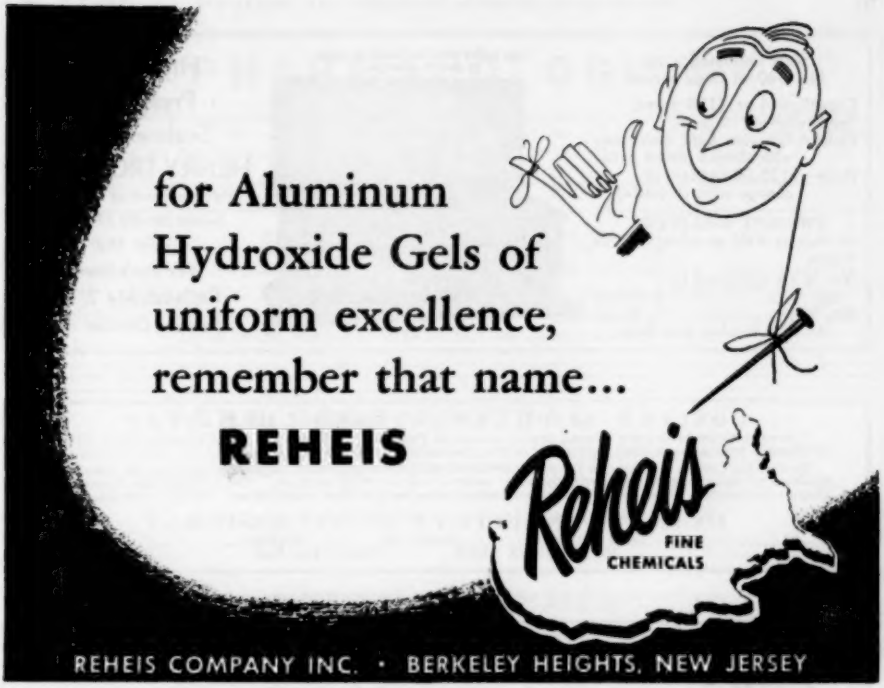

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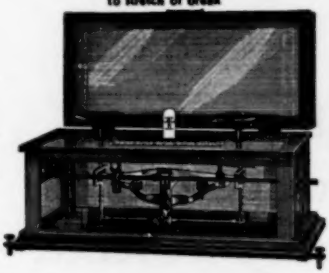

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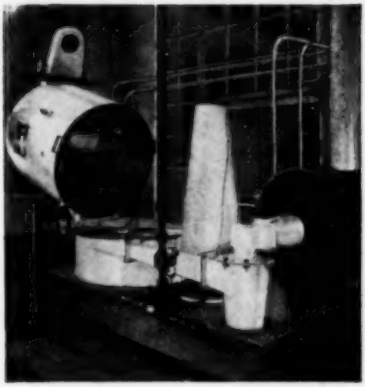

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Peroxide Test Method for Determining Oil and Fat Stability*

By MILTON J. GOLDEN

Most of the methods recommended for determining oil and lard stability have been found to be time-consuming and unreproducible. A simple, accurate, and reproducible peroxide test method is proposed for determining the stability of vegetable oils and lard. A saving of as much as 75–90 per cent of the time required by the Swift stability method is made possible by carrying out the test at 125°. A definite and satisfactory correlation exists between the values obtained by the proposed peroxide method at 125° for oils and lard and those by the active oxygen method at 97.7°.

THE atmospheric oxidation of oils and fats with the subsequent development of rancidity has been, and still is, of major economic and industrial importance. As a result, the use of antioxidants for the preservation of these products has become more and more widespread. Considerable attention has also been given to the evaluation of these agents to determine their effectiveness in inhibiting and retarding rancidity.

The unpleasant odor and taste, characteristic of rancid oil and fat, are due to the oxidation products formed by the action of atmospheric oxygen on the unsaturated glycerides. This type of oxidation, known as autoxidation, is accelerated by both light and heat.

In the initial stages of autoxidation, an induction period is believed to exist, characterized by the gradual accumulation of organic peroxides and hydroperoxides (1). These peroxides are eventually converted into hydroxy acids, hydroxy keto acids, lactones and other intermediate products such as aldehydes which are responsible for the characteristic odor and taste of rancid oils and fats. After the appearance of a rancid odor, indicating the end of the so-called induction period, oxidation seems to proceed more rapidly with the formation of a complex mixture of products.

Some vegetable oils are known to have rather long induction periods and, therefore, fairly good keeping quality, which may be due in part to the presence of such natural inhibitors as tocopherols and other complex phenolic compounds that retard peroxide formation. Modern refining and processing tend to remove some of these natural antioxygenic substances, and, as a result, the refined oil is less resistant to oxidative rancidity than the crude oil.

* Received September 12, 1950, from the Research Laboratories, McKesson & Robbins, Inc., Bridgeport, Conn.

In order to retard this oxidation process, it has been the custom to add certain antioxidants. Such compounds as hydroquinone, pyrogallol, catechol, and other related phenolic chemicals have been frequently used in nonedible products. However, they have been found to be unsuitable for general use, in view of the fact that they possess toxic properties and tend to discolor the material into which they are incorporated.

In the past few years, many other chemical compounds have been suggested for use as antioxidants in foods and edible products. Of these compounds, gallic acid, propyl gallate, nordihydroguaiaretic acid, diisoeugenol, and 5-pentadecyl resorcinol seem to be the ones most commonly used today.

For evaluating these compounds according to their antioxidant properties and for detecting actual and impending rancidity, several test methods have been suggested. After careful examination of these methods, most of them have been found to be either impractical or unreproducible.

In the incubator test for detecting rancidity (2), fats are exposed to air at elevated temperatures and then examined organoleptically at regular intervals for the development of a rancid odor. This test has been found to be objectionable in view of the time involved and the results which are dependent entirely on individual judgment.

The time required by a fat to absorb a certain amount of oxygen has also been made the basis for determining keeping quality (3, 4, 5). This method requires complicated and expensive apparatus and gives results that are influenced by the presence of oxidation products and variations in absorption rate due to changing pressure.

A test depending upon the collection of the volatile aldehyde products from decomposed, incubated fats and the quantitative determination of these aldehydes has served as an index of the keeping quality of fats. Since a certain rancid point, depending upon the amount of aldehydes collected, is not specific for all fats, serious errors may arise in grading fats on this basis. Furthermore, the test is difficult to carry out and is not sufficiently sensitive to detect small differences.

The quantitative determination of peroxides in oils and fats iodometrically seems to be, at present, the most common method in use for determining and measuring stability. The original iodometric test suggested by Lea (6) has been modified and extended by Wheeler (7) and other investigators (8, 9).

This method was devised for the purpose of measuring the keeping quality of lard. Now it has been applied to all fat and oil products where stability is important. It is impossible, at times, to obtain reproducible quantitative values with this method, irrespective of the precautions taken, thus making it necessary occasionally to use the shelf-storage test and determine the development of rancidity organoleptically.

The need for an improved method for determining the stability or keeping quality of oils and fats has been emphasized repeatedly. King, Roschen, and Irwin introduced the Swift stability test (8) in which samples of oils and fats are aerated at 97.7°. The time required to obtain predetermined peroxide levels and other symptoms of rancidity is taken as a measure of their keeping quality. While this method has not found complete acceptance, it seems to be the predominant one in use today, as far as the edible fat industry is concerned.

Although the Swift stability test, also known as the active oxygen method (AOM), has been considered satisfactory for lard, it has been found to be somewhat unsuitable for vegetable oils. Occasionally the iodometric active oxygen method values do not represent the true breaking point for vegetable oils. Actual shelf-storage tests on some vegetable oils are known to be considerably longer than the time indicated by the active oxygen method.

Various modifications of the Swift stability method have been introduced. King, *et al.* (8), studied the catalytic action of various metals in contact with oil samples during incubation. They found that certain metals, such as copper and zinc, accelerated peroxide development and progress of rancidity, but the results varied considerably with the different oils and were markedly influenced by antioxidants.

Higher temperatures have also been used as a means of speeding the breakdown of oils and fats. Freyer (10) in 1935 suggested the advantages from the use of higher temperatures. Mehlenbacher (11), using the iodometric method, studied the effect of elevated temperatures (97.7, 110, and 120°) on the rate of rancidity development in animal and vegetable fats. At 120°, he reported the correlation to be poor, attributing this to the failure of the peroxide formed to be reduced by the HI, and the probable rearrangement and formation of peroxides different from those at 97.7°. At 110°, fairly good correlation was obtained, accompanied with an appreciable decrease in the time required to carry out the test.

In view of the fact that the Swift stability test and all its modifications have several interfering factors and shortcomings, a new stability test method is advisable, provided it can eliminate most of the interferences inherent in the iodometric method and make possible a considerable saving of time to complete the test.

In spite of the fact that there seems to exist among research workers the policy or aim to only modify existent data, an entirely new method is sometimes not only advisable, but even necessary.

The purpose of this paper is to introduce a new and rapid peroxide test method for determining oil and fat stability, to show the suitableness of the ferrous thiocyanate reagent as a means of evaluating and comparing antioxidants with a considerable saving of time, and to show that a correlation exists between the values obtained by the ferrous thiocyanate method and those by the active oxygen method.

EXPERIMENTAL

The use of ferrous thiocyanate reagent in the estimation of peroxides in mineral oils (12), fats and vegetable oils (13), and hydrocarbons (14) has been known for several years. It has been used in the development of the peroxide test for mineral oil (12) and petrolatum (15) and in determining the peroxide content of milk and butterfat (16). The ferrous thiocyanate reagent has already been reported to be of value in the estimation of peroxides in oils and fats, and the values obtained have been found to be proportional to the iodometric AOM values (13).

The facts and reactions as described in the peroxide test for mineral oil were used in developing the following proposed method: $^1/_2$-cc. samples of oils heated at 125° were removed at half-hour intervals, with the subsequent addition of 5 cc. of a test solution consisting of ferrous sulfate, ammonium thiocyanate, sulfuric acid, reduced iron filings, and iron wire. The developing color, which varied from pink to dark orange or red, depending upon the amount of peroxides formed by the breakdown of the oil and fat, was observed. The first tube having a perceptible rancid odor and exhibiting a marked change in color from the pink to dark orange or red after shaking with the test solution is regarded as the breaking point expressed in hours.

Apparatus.—One-liter round-bottom flask and reflux condenser; test tubes 1 in. by 6 in. marked for 5-cc. filling; constant temperature oil bath; Swift stability keeping test apparatus.

Materials.—Ferrous sulfate, C. P.; ammonium thiocyanate, C. P.; iron by hydrogen, Merck (reduced iron); iron wire No. 30, for standardization; acetone, U. S. P.

Preparation of Test Solution.—In a round-bottom, wide-mouthed flask, 2 Gm. of ferrous sulfate is dissolved in 100 cc. of distilled water containing 2 cc. of concentrated sulfuric acid. Two hundred cubic centimeters of acetone and 100 cc. of a 2% aqueous solution of ammonium thiocyanate are then added to the above solution. Iron powder, 0.2 Gm., and 6 in. of No. 30 iron wire are finally added to reduce any trace of ferric ion present. The resulting mixture is refluxed on a steam bath until the red color of the solution has disappeared. This reduction takes approximately two hours, it being necessary to continue refluxing to insure lack of color.

Fresh solution is prepared on the day on which the test is to be carried out. However, it is possible to preserve it in a colorless state by careful bottling with carbon dioxide.

Since it is necessary to avoid any contact with air, a method has been devised to dispense this solution. A siphon is passed into the upper end of the reflux condenser through a two-hole cork stopper. The second hole is fitted with a glass stopcock, which serves as a vent. Carbon dioxide is admitted through the vent to inaugurate the flow of the solution, the pressure forcing the liquid into the siphon, at the end of which is attached a glass stopcock to withdraw the solution.

Procedure.—Test tubes containing 5-cc. samples of oil or lard, which have been previously heated to 90° by immersing in hot water, are placed in an oil bath regulated at 125° ± 1°. The samples in the tubes are then aerated according to the Swift stability method with approximately 2.33 cc. of air being passed through each per second.

At half-hour intervals, $^1/_2$-cc. samples are removed and added to test tubes graduated to 5 cc. After several samples have been collected, 5 cc. of the colorless test solution is added to each tube which has previously been heated to approximately 70°. After the removal of the air by means of carbon dioxide, the tube is tightly stoppered with a cork and then shaken by hand for fifteen seconds. The formation of additional peroxides in the samples is detected by the development of a dark orange or red coloration of the lower layer in the test tubes. The first tube having a perceptible rancid odor and exhibiting a marked change in color from the initial pink to dark orange or red after shaking with the test solution is regarded as the breaking point expressed in hours.

DISCUSSION

In view of the unsatisfactory results frequently obtained for oils by the active oxygen method, the time required to produce both a noticeable increase in peroxide content per kilogram of oil and a perceptible rancid odor is suggested as the true breaking point for oils. The proposed 20 milliequivalent per kilogram level was used only for lard.

In order to determine whether any correlation exists between the values obtained by the peroxide test method at 125° and the active oxygen method at 97.7°, the samples under study were tested according to the stability method of King and his associates (8). For additional comparative purposes, the peroxide test method was also carried out at 97.7°.

Table I shows how closely the peroxide test method values at 97.7° for peanut and cottonseed oils agree with those of the modified active oxygen method at 97.7° and how the peroxide test values at 125° are proportional to the modified AOM

TABLE I.—COMPARISON OF THE STABILITY VALUES OF THE PEROXIDE TEST METHOD FOR VEGETABLE OILS WITH THOSE OF A MODIFIED ACTIVE OXYGEN METHOD

Sample	Swift Stability, Modified 97.7°, Hr.	Peroxide Test, 97.7°, Hr.	Peroxide Test, 125°, Hr.	AOM (Mod.) Accelerated Time, 125°
Cottonseed Oil	5	5	1¼	4
+0.1% propyl gallate	38	39	9	4.2
+0.1% NDGA[a]	20	18	5	4
+0.1% tertiary-butylhydroquinone	30	30	7½	4
Peanut Oil	5	5	1	5
+0.1% propyl gallate	66	68	13	5
+0.1% NDGA[a]	42	44	8½	4.9
+0.1% tertiary-butylhydroquinone	26	28	5	5.2

[a] Nordihydroguiaretic acid.

values. The effectiveness of three well-known antioxidants incorporated into the samples at a concentration of 0.1% was also compared. The values reported represent the average of at least three separate determinations deviating not more than ±2%.

TABLE II.—COMPARISON OF THE STABILITY VALUES OF THE PEROXIDE TEST METHOD FOR LARD WITH THOSE OF THE ACTIVE OXYGEN METHOD BASED ON THE 20 M. E./KG. LEVEL

Sample	Swift Stability, 97.7°, Hr.	Peroxide Test, 97.7°, Hr.	Peroxide Test 125°, Hr.	AOM Accelerated Time, 125°
Lard	3	3	2/10	10
+0.1% propyl gallate	122	116	12	10.1
+0.1% NDGA[a]	53	50	5½	9.7
+0.1% tertiarybutylhydroquinone	105	90	10	10.5

[a] Nordihydroguiaretic acid.

Table II compares the results of the peroxide test method for lard with those of the active oxygen method on the basis of 20 M. E./Kg. Only fair correlation was found to exist at 97.7° between the peroxide test and the AOM values. However, the sensitivity of the proposed method was increased severalfold with satisfactory correlation when the peroxide test was carried out at 125°.

This accelerated peroxide test method makes possible a saving of as much as 75-90% of the time previously required to carry out the test. The average ratio of the time required at 97.7° for the active oxygen method to break down the samples, with and without antioxidants, to the accelerated time required at 125° for the peroxide test method is estimated to be approximately 4 for cottonseed oil, 5 for peanut oil, and 10 for lard with a maximum deviation of ±0.2 for the oils and ±0.5 for lard.

If it is desirable to report the results on the active oxygen method basis, then it is only necessary to multiply the number of hours obtained at 125° by 4 for cottonseed oil, by 5 for peanut oil, and by 10 for lard in order to obtain the hours that would have resulted at 97.7°.

The results obtained by this proposed peroxide test method show unquestionably the usefulness of this simple, reproducible, and accelerated method in evaluating and comparing the stability of oils and lard, and the effectiveness of the several antioxidants recommended for retarding rancidity.

This suggested peroxide test method offers a means for determining with considerable accuracy the stability of oils and lard with and without antioxidants macroscopically, and makes possible a saving of as much as 75-90% of the time previously required. It can undoubtedly replace the troublesome and time-consuming active oxygen method.

SUMMARY

Most of the methods recommended for determining oil and lard stability have been found to be unreproducible, time-consuming, and impractical.

A simple, rapid, and reliable peroxide test method is proposed for determining the stability of oils and lard with and without antioxidants.

A saving of as much as 75–90 per cent of the time previously required is made possible by carrying out the peroxide test at 125°.

A definite and satisfactory correlation has been found to exist between the values obtained by the peroxide test method at 125° for vegetable oils and lard, and those by the active oxygen method at 97.7°.

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A Pharmaceutical Study of Jelene Ointment Base*,†,‡

By SHIRLEY FOSTER, D. E. WURSTER, T. HIGUCHI, and L. W. BUSSE

Physical properties including viscosities over wide temperature ranges, miscibility with various pharmaceuticals, and stability of Jelene, a new hydrocarbon ointment base, have been studied. A bacteriological study utilizing the agar cup-plate method in which this ointment base was compared with petrolatum, cold cream, and vanishing cream was performed. The results of an investigation on the rate of release of certain drugs from the base are reported. These studies show that Jelene has many desirable properties as an ointment base.

JELENE ointment base is a combination of mineral oils and heavy hydrocarbon waxes, having a molecular weight of approximately 1,300, the large proportion of which are liquid and are retained in what is believed to be a matrix of sub-microscopic interstices. It is believed that the unusual properties of this hydrocarbon base can be attributed to the mobility of the liquid phase which makes possible internal migration, and as a result, a slow but constantly changing interface. This property of presenting a changing interface is extremely important, and one which encourages the diffusion of a drug into the surrounding medium.

PHYSICAL PROPERTIES

Jelene ointment base is a soft, smooth, homogeneous, neutral and nonirritating ointment base. It maintains a desirable consistency over a wide temperature range (−15 to 60°) because of its relatively low thermal coefficient of viscosity. This means that it will not liquefy at temperatures occurring in tropical climates and will not harden excessively when exposed to relatively low temperatures. While Jelene does not liquefy at body temperatures, its viscosity is such that it is easily applied to the skin. Figures 1–6 illustrate the viscosity properties of Jelene ointment base over a wide temperature range, as compared to that of petrolatum.

Further evidence illustrating the desirable viscosity properties of Jelene ointment base as compared with petrolatum are shown in Figs. 2–6, prepared from data taken during cone-penetrometer studies. Jelene ointment base is not miscible with water and is therefore hydrophobic in character. Yet it is easily removed from the skin since it is readily absorbed by cloth or cleansing tissue. When the base is removed in this manner, the skin to which it was applied possesses a velvety feel and is not greasy. When water-miscible properties are desired, Jelene may readily be made hydrophilic by the incorporation of low percentages of hydrophilic agents such as the glyceryl or sorbitol monolaurates or oleates or the various polyoxyethylene sorbitan esters, etc.

PHARMACEUTICAL STUDIES

The fusion process cannot be employed when Jelene is the ointment base, since the fusion temperature causes a disruption of the gel structure which does not completely reform during the cooling process. However, the fact that Jelene ointment base maintains its viscosity at elevated temperatures means that the addition of viscosity-inducing substances, such as beeswax, ceresine, or paraffin, is not required and therefore the fusion process is actually eliminated as a compounding procedure when this base is used.

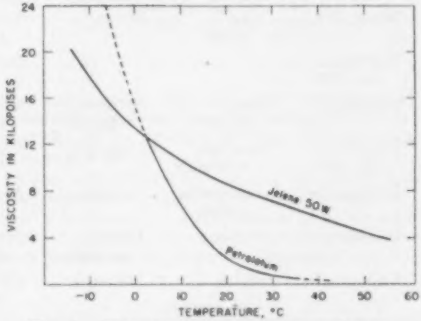

Fig. 1.—Viscosity of Jelene and petrolatum.

Most substances used in present-day dermatologic practice are readily incorporated into Jelene ointment base. The following is a partial list of substances which were readily incorporated without fusion into smooth homogeneous ointments using light liquid petrolatum as the levigating medium.

Ammoniated mercury	Belladonna extract
Benzoic acid	Boric acid
Chrysarobin	Iodine
Menthol	Methyl salicylate
Penicillin	Phenol
Salicylic acid	Sulfadiazine
Sulfathiazole	Sulfur
Tannic acid	Zinc oxide

Inasmuch as Jelene ointment base is purely hydrocarbon in nature and therefore relatively chemically unreactive, few chemical incompatibilities

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† This paper is abstracted from a thesis presented to the graduate school of the University of Wisconsin by Shirley Foster in partial fulfillment of the requirements for the degree of Master of Science.
‡ Jelene ointment base is a product of the Research Products Corporation, Madison 10, Wisconsin.

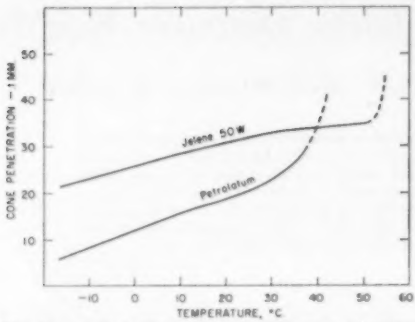

Fig. 2.—Cone penetration *vs.* temperature of Jelene and petrolatum.

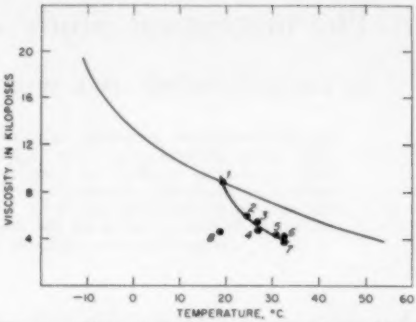

Fig. 3.—Effect of milling (paint type) on Jelene ointment base (viscosity *vs.* temperature).

will be encountered. However, like petrolatum, Jelene is nonpolar and is immiscible with polar liquids such as water, alcohol, and glycerin.

A series of aging tests, in order to determine the stability to time and temperature, were carried out in which ointments prepared from the commonly used ointment bases were compared. The data so derived are summarized in Table I.

The results of the above tests illustrate very definitely the stability of Jelene ointments at elevated temperatures. This is due to the unique structure of the Jelene base which permits it to retain its viscosity at these temperatures.

Since ointments are subjected to milling action in the course of their manufacture, the ability of Jelene ointment base to maintain its structure when subjected to this process was investigated. The data are shown in Fig. 3.

The data in Fig. 3 show that the viscosity of Jelene decreases significantly after one milling and

TABLE I.—AGING TESTS, 2-OZ. OINTMENT JARS, 50°, FIFTY DAYS

Bases	Coal Tar, 2%	Iodine, 7%	Phenol, 2%	Sulfathiazole, 5%	Whitfield's Ointment	Zinc Oxide, 20%
Jelene 50W	Bled 2 drops	Stable	Bled a trace	Stable	Stable	Bled a trace
Carbowax No. 1540	Base liquefies below 50°					
Aquaphor	Bled a trace	Softened slightly	Bled considerably	Stable	Stable	Bled considerably
U. S. P. hydrophilic	Bled considerably	Liquefied	Foamy, soft bleeding	Bled considerably	Phase separation	Bled considerably
Hydrosorb[a]	Phase separation	Liquefied	Phase separation	Phase separation	Separated	Separated
Petrolatum	Stable	Iodine separated	Not homogeneous	Stable	Some separation	Settled out
Unibase	Stable	Softened	Phase separation	Stable	Phase separation	Stable

[a] Base liquefies below 50°.

TABLE II.—BACTERIAL INHIBITION STUDIES AGAR CUP-PLATE METHOD *S. aureus* 209p

Medicament	Per Cent	Zone of Inhibition in Millimeters				
		White Pet.	Jelene	Cold Cream	Jelene O/W (60%)	Vanish. Cream
Boric acid	10	No zone	No zone	No zone	54.6	40.3
Chlorothymol	2	47	50	24	40	55
Ammoniated mercury	5	29.8	35.5	29.6	50	39
Mercuric chloride	1	57	60	43	58	37
Mercuric oxide yellow	1	28.3	29.6	29	38	33.6
Mercurous chloride	30	26	29	31	70.6	40
Phenol	2	No zone	19	19	43	27
Penicillin	0.1	28.5	40	38	81.5	58
Sulfathiazole	5	No zone	No zone	No zone	38	38

is not greatly affected by five additional millings in the paint-type mill. The final viscosity after cooling to room temperature is 4,400 poises or 440,000 centipoises, a viscosity well within the desirable range for ointment bases.

BACTERIOLOGICAL STUDIES

Bacteriological studies were carried out in order to compare the relative effectiveness of certain medicaments when incorporated into Jelene ointment base, Jelene hydrophilic, petrolatum, a cold cream, and a vanishing cream. The agar cup-plate method was employed using standard bacteriological procedures throughout, and *Staphylococcus* 209p as the test organism. The width of the zone of inhibition in mm. is a measure of the rate of release of the drug from the ointment base involved and indicative of the rate of diffusion of the drug into an agar medium. Table II shows the results of these tests.

An analysis of the data in Table II shows that Jelene ointment base and Jelene hydrophilic ointment base are equal or superior to petrolatum vanishing cream and cold cream as ointment bases for the medicaments tested as to the rate of release of the medicament into an agar medium.

For those compounds which are quite insoluble in water and which should be incorporated into oil-in-water emulsion bases, Jelene hydrophilic ointment base was superior to a regular vanishing cream formula. These facts, in addition to the superior physical properties, would indicate the Jelene ointment bases to be the ointment bases of choice for these medicaments.

RATE OF RELEASE STUDIES

Although it is difficult to evaluate the rate of release of medicaments from purely *in vitro* studies, it is felt that the results of the following procedure may have some significance. The data obtained are shown in Figs. 4, 5, and 6.

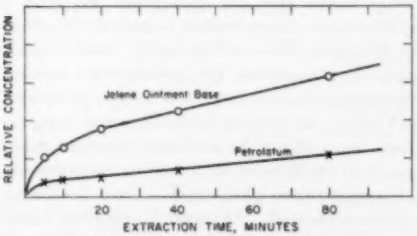

Fig. 4.—Salicylic acid (5%).

Experimental Procedure.—The ointment bases were packed into weighing bottle caps (20 mm. diam.), holding approximately 5 Gm. of ointment. These containers were then placed in individual 250-ml. Erlenmeyer flasks, together with 75 ml. of boiled distilled water. These were then placed in a modified rotary Warburg apparatus, such that the flasks were kept at a constant temperature (25°) and subjected to constant oscillation of 120 cycles per minute. Samples of the solution were periodically withdrawn and analyzed spectrophotometrically for the amount of drug extracted from the ointment bases.

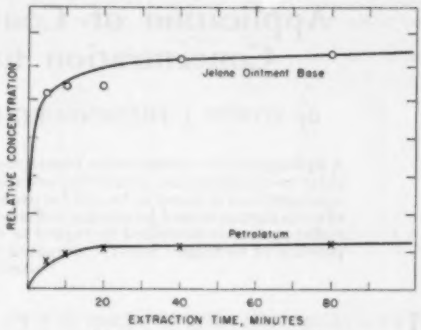

Fig. 5.—Sulfanilamide (5%).

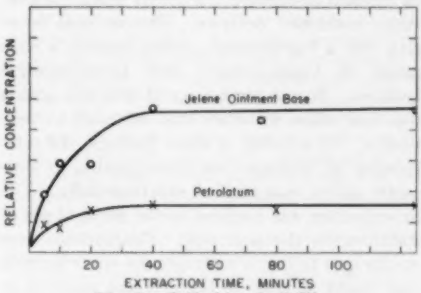

Fig. 6.—Sulfathiazole (5%).

It is evident from the data in Figs. 4, 5, and 6 that of the substances tested, Jelene ointment base appears to be superior to petrolatum as to the amount of drug it releases at an oil-water interface, and substantiates the results of the bacteriological studies.

SUMMARY

An analysis of the data obtained from the viscosity determinations and the stability tests shows that ointments prepared with Jelene ointment base possess unusual viscosity characteristics and remarkable stability properties over a wide temperature range.

On the basis of the *in vitro* studies (bacteriological and physical) carried out, Jelene ointment bases appear to permit a greater release of the incorporated drug, when exposed to an oil-water interface, than petrolatum. This would indicate that higher concentrations of the drug in the tissue fluids would be obtained from Jelene ointment bases when these are applied to subcutaneous or mucous membranes.

Application of Controlled Hydrogen-Ion Concentration for Antiseptic Use*

By EDWIN J. FREDENBURGH† and BENJAMIN P. HECHT‡,§

A hydrogen-ion concentration corresponding to *p*H 3.5 is shown to exhibit bactericidal to bacteriostatic properties toward pathogenic bacteria. This hydrogen-ion concentration is found to be safe for use in topical application. Greater antibacterial effect is demonstrated by using a buffer to maintain the *p*H. The composition of the buffer system is described in regard to base and acid components. The antiseptic potency of hydrogen ions is compared with the antibiotics and several other antibacterial agents.

IT WAS OBSERVED by one of the authors (E. J. F.) that the substrate on which a strain of *Penicillium steckii* Zaleski was grown acquired an unusual antibacterial property when the substrate contained dextrose. The unusual property was a bacteriostatic action against a wide range of Gram-positive and Gram-negative bacteria. It was further noted that this action was lost when substrate was adjusted to neutrality. As a result of these findings, the relationship of hydrogen-ion concentration to antiseptic action was studied experimentally. The investigation was confined to the effect of *p*H of solutions on the acid side. The authors were encouraged to find a hydrogen-ion concentration that might be toxic to bacteria and nontoxic to tissues, and then to find a buffered system that would maintain such a hydrogen-ion level. The creation of such an antiseptic environment could then be applied to the control of local infections. The maintenance of *p*H below 5 for a specified period of time to allow for the establishment of a state of antisepsis has already been noted (1), while use of buffered acids also has been reported (2).

Both organic and inorganic acids are known to exhibit antibacterial properties (3). Perhaps the earliest study on the antibacterial action of acids in light of the theory of electrolytic dissociation of acids dates back to the work of Krönig and Paul (4), who related the antibacterial characteristics with the degree of ionization of acids. Later studies (5) also have attributed the action of the mineral acids to the hydrogen-ion concentration. Many organic acids, however, exert greater antibacterial action than accounted for by the hydrogen-ion concentration. It is deduced that the undissociated acid present accounts for this effect. In general, the action of organic acids is described as specific for the acid (5).

Also, it is known that culture growth is dependent upon the maintenance of optimum *p*H, among other factors, and that by altering the *p*H, this growth may be inhibited or inactivated. This limiting *p*H has been determined for a large number of bacteria (5–7). Many bacteria do not grow at *p*H 5, and apparently, with very few exceptions, no bacteria can survive and grow at *p*H 3.

The authors recognize the general practical importance of the resolution of acidity into the concentration of hydrogen ions and the quantity of acid capable of furnishing this ionized hydrogen, in order to effect sustained antiseptic action. The acid selected as ideal for this application would give the appropriate *p*H with a hydrogen-ion concentration essentially constant for all concentrations of the acid. Such an acid, ideal for maintaining the hydrogen-ion concentration at a constant level, is largely hypothetical, and in fact, at variance with the law of mass action. An acid has the ionization constant maintained at equilibrium by a shift in the hydrogen-ion concentration when changes occur in concentration of the acid. However, by means of buffer action, an acid with a dissociation constant of proper magnitude can be modified to yield a preparation that is both suitable and effective.

Protection afforded by the buffer action against changes in *p*H is held essential, for unbuffered acids fail to be effective under all conditions of use in antisepsis. Substances naturally present in the wound, such as pus and serum, as well as basic products of bacterial metabolism, readily neutralize the acid to alter the *p*H from the more

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§ The authors are indebted to Dr. Walter W. Jetter, Professor of Legal Medicine, Boston University School of Medicine, and Dr. John B. Hamblet, Pathologist, Manchester Memorial Hospital, Manchester, Conn., for valuable suggestions. To Miss Mildred V. Masse and Miss Mary F. O'Donnell the authors express appreciation for the assistance given in the preparation of this manuscript. The authors are also grateful to the Emerson Hospital, Concord, Mass., and Selenium Research Products, Ipswich, Mass., for use of facilities.

effective range. In such circumstances only an increase in pH is evident where the initial concentration of hydrogen ions in the acid solution is in the order of 10^{-3} molar. The kind of buffer action employed, therefore, need function only against a decrease in hydrogen ions.

EXPERIMENTAL

Useful Range of Hydrogen-Ion Concentration for Antisepsis.—Although greater antibacterial activity follows increased hydrogen-ion concentration, consideration must be given to the toxicity of this ion to tissues. The optimum effective concentration is therefore the maximum concentration of hydrogen ions that the tissues can withstand, with, of course, adequate margin of safety provided.

Several methods were used to establish a safe pH for use in antisepsis. Table I shows the effect of instilling solutions of various degrees of acidity into the eyes of guinea pigs. Solutions of pH 1.0 were toxic; those of pH 2.0 produced no apparent irritation. In Table II, indicating the effect of subcutaneous injections, toxicity was apparent for a solution of pH 2.0, but not for solutions with higher pH. Toxicity findings were confirmed by continuous application of a buffered solution of pH 3.2 to ulcerated cutaneous areas of human subjects. Signs of irritation or discomfort were not manifest. Further corroboration was made by studying the discomfort produced in the eyes of normal human subjects. The discomfort generally experienced with the buffered solution of pH 3.2 was of slightly shorter duration than that caused by an ophthalmic solution of sodium sulfacetimide (Schering Corp.), although capillary dilatation of brief duration occurred with the former solution.

For use in antisepsis, a pH of about 3.5 was consequently selected to insure safety to tissues, although a lower pH can evidently be tolerated by skin and mucous membrane tissues.

TABLE I.—RELATIONSHIP OF pH TO IRRITATION PRODUCED IN EYES OF GUINEA PIGS

pH of Isotonic Solution[a]	Frequency of Instillation of Solution	Result[b]
1.0	Two drops every 1/2 hr. for 2 1/2 hr.	Irritation of conjunctiva produced after first instillation
2.0	Two drops every 1/2 hr. for 5 hr., followed by 2 drops every 10 min. for 1 hr.	Irritation not observed
3.0	Same	Irritation not observed
4.0	Same	Irritation not observed
5.0	Same	Irritation not observed

[a] This solution contained 0.9% sodium chloride with hydrochloric acid used for the adjustment of the pH. Recent literature shows this salt concentration to be isotonic with lacrimal fluid [Krough, A., Lund, C. G., and Pedersen-Bjergaad, K., *Acta Physiol. Scand.*, **10**, 88(1945); through *Chem. Abstr.*, **40**, 1921(1946)].

[b] Five to seven animals were used to test the effect of each pH. One eye of each animal was treated, while the other eye served as control. Irritation phenomenon was carefully watched for, following each instillation, and the period of observation extended to at least twenty-four hours.

TABLE II.—RELATIONSHIP OF pH TO IRRITATION PRODUCED BY SUBCUTANEOUS INOCULATION IN GUINEA PIGS

pH of Isotonic Solution[a]	No. Animals Used	Per Cent of Animals to Develop Tissue Necrosis		
		1st Day	2nd Day	7th Day
1.0	5	40	80	80
2.0	4	0	50	50
2.5	4	0	0	0
3.0	6	0	0	0

[a] Adjustment of normal saline solution was made with hydrochloric acid to produce the isotonic solution at the above pH values. Inoculation was made using 2 ml. of solution.

Selection of Buffer System for Use in Antisepsis.—The successful application of hydrogen ion for antisepsis depends upon effective control of pH in the established range of about 3.5. A suitable buffer was therefore devised. The buffer developed contains free, weak acid which generally constitutes in part buffer systems, but, in addition, this buffer includes a very weak base in the form of salts of the weak acid. Alkaline substances that come in contact with this buffer are not only neutralized by the weak acid present, but also by interchange of cations, unlike conventional buffer systems. In this manner a buffer system is produced with additional buffer capacity.

Peptone (Bacto-Peptone) was selected for the very weak base in the buffer system because it has an ionization constant much smaller than that for ammonium hydroxide, indole, and other amine products of bacterial metabolism. Peptone neutralizes basic compounds by a reaction formulated as follows:

$$\text{Peptone Salt} + \text{Base} \rightarrow \text{Peptone} + \text{Salt of Base}$$
$$(K_b > 10^{-10}) \quad (K_b < 10^{-10})$$

Peptone used in our experiments is not a single substance, but a complex of proteoses, peptones, amino acids, and other degradation products of enzymatic hydrolysis of proteins (8). The dissociation constants, K_b, of these constituents, however, are all of low magnitude which we have established as $<10^{-10}$, based upon a negative exchange reaction of cations when peptone and aniline chloride were dissolved together. The ionization constant for the latter base is 3.83×10^{-10} at 25°.

The buffer action of peptone was observed to extend in the pH range 4.2 to 4.9. This was demonstrated by titration of acid (Fig. 1) in the presence of peptone. Greater effectiveness of buffer capacity would result for the intended application if the buffer action of peptone extended to lower pH Nevertheless, the use of peptone in the buffer product is both advantageous and expedient, as bacterial growth is generally inhibited between pH 4 and 5. Peptone therefore affords additional buffer capacity nearly superimposing the buffer action accounted for by the presence of weak acid in the buffer system.

Further demonstration of buffer action of peptone is provided by the comparison of the relative slopes of titration curves for solutions of gluconic acid and gluconic acid with peptone gluconate (Fig.

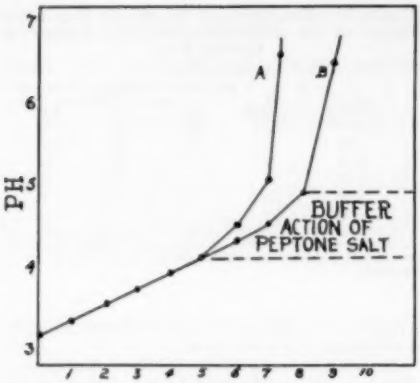

Fig. 1.—Buffer action of peptone. 100-ml. volumes of 2% gluconic acid, adjusted to *p*H 3.2 with sodium hydroxide or peptone each to form: (*A*) sodium gluconate-gluconic acid buffer, and (*B*) peptone gluconate-gluconic acid buffer, respectively, were titrated to show special buffer action of Bacto-Peptone.

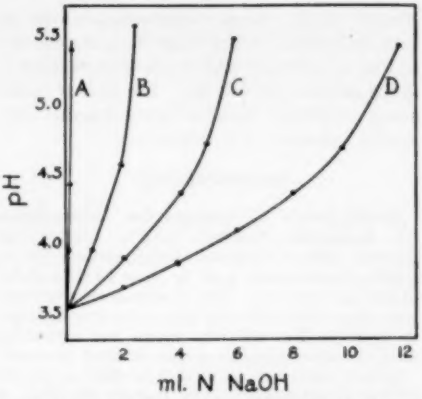

Fig. 2.—Relationship of buffer capacity to peptone concentration in gluconic acid buffer. (*A*) gluconic acid; (*B*) gluconic acid with 1% peptone; (*C*) gluconic acid with 2.5% peptone; (*D*) gluconic acid with 5% peptone. Titration curves were obtained by neutralization of 100-ml. volumes of the unbuffered and buffered gluconic acid solutions each with initial *p*H of 3.5

2). The slopes of these curves also express the relationship of buffer capacity to peptone concentration. For the concentration range of 1 to 5% peptone tested, a direct proportionality between concentration and buffer capacity is concluded. Considerable variation exists, however, in the buffer qualities of the various commercial peptone products. A sample of Merck peptone exhibited rather weak buffer action, whereas Difco Bacto-Peptone produced uniformly good buffer action for several different batches examined.

The choice of peptone as the base was also dependent upon its solubility, stability, diffusibility, and lack of toxicity.

Selection of an acid for inclusion in the buffer system comprised a very important consideration determining, in part, buffer range. Furthermore, the ratio of salt to acid must be considered in relationship to the degree of ionization of the acid compound employed. Maximum buffer capacity is attained when dealing with an acid of appropriate strength if log (Salt)/(Acid) equals 0. However, for the intended application of the product, greater buffer capacity was effected by reducing the log (Salt)/(Acid) ratio to −0.5, as the only possible direction of change in *p*H is from decreasing hydrogen-ion concentration. Figure 3, showing calculated titration curves as theoretical approximations, clarifies the significance of salt to acid concentrations. These titration curves determine that the greatest buffer capacity results when the (Salt)/(Acid) ratio is set at the initial end of the flat portion of the titration curve where, approximately, log (Salt)/(Acid) equals −0.5. The dissociation constant of the buffer acid for this application may now be computed from the Henderson-Hasselbalch equation, *p*K equals *p*H − log (BA)/(HA) by setting log (BA)/(HA) equals −0.5.

Referring again to the titration curves in Fig. 3, comparison is made of the relative buffer capacities for buffers of *p*H 3.5 employing acids of *p*K 4.0 and *p*K 3.5 to give the described (Salt)/(Acid) ratios. These two buffers possess identical buffer capacities in the *p*H region 3.5 to 4.1, but there is considerable extension of buffer capacity for the buffer of lower (Salt)/(Acid) ratio in the *p*H region 4.1 to 5.0. In terms of equivalent base, the latter buffer yields more than 100% greater buffer capacity for the *p*H range of 4.1 to 5.0. For the buffer product application, therefore, an acid compound with an ionization constant of Ka equals 10^{-4} is preferred to prepare the buffer of *p*H 3.5.

δ-Gluconolactone, which on hydration in solution forms gluconic acid in equilibrium with γ- and δ-lactones (9), was used in these experiments with peptone to compose the buffers. The ionization constant of the acid (10) is 2.5×10^{-4} at 25°, which is greater than the calculated theoretical value of 1×10^{-4} for optimum buffer capacity. Selection

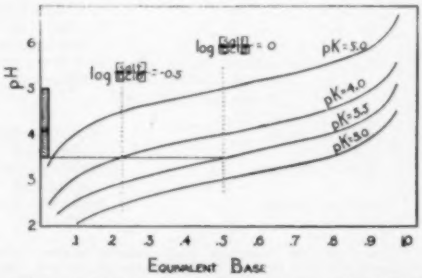

Fig. 3.—Theoretical titration curves of acids of *p*K values indicated. Calculations are based on expression *p*H equals $pK + \log \frac{(BA)}{(HA)}$, and complete ionization of the salt formed in the titration is assumed.

of this particular acid was made because of the reasonable certainty that the undissociated acid was entirely devoid of pharmacological[1] and bacteriological[2] action, and any antibacterial effect noted could be entirely attributed to hydrogen ions alone. Selection of this acid was also dependent upon other desirable physical and chemical properties of the acid, particularly its ready solubility, stability, and lack of appreciable vapor pressure which otherwise would lead to irritation from acid vapors in practical use.

Blood serum was added to peptone-gluconic acid buffer, pH 3.5, to determine the stability of this acid buffer under conditions naturally encountered. Serum constitutes a very efficient buffer system, containing bicarbonate, basic phosphate, and protein compounds that will react with acid buffers. Therefore, the use of serum offered a means to evaluate the effectiveness of the acid buffer to maintain its hydrogen-ion concentration in the antibacterial range. Fresh, pooled, human blood serum, pH 7.7, was added in 1:1 ratio to the acid buffer, containing 5% peptone, and resulted in a change in pH reading from 3.5 to 4.1. Growth of pathogenic bacteria is generally inhibited at pH 4.1.

The buffer product applied to the bacteriological work which follows was prepared from 1 and 5% Bacto-Peptone (Difco Laboratories, Inc.) as the base constituent, with gluconic acid (Chas. Pfizer & Co.) added to adjust the hydrogen-ion concentration to pH 3.5.

Bacteriological Study of Buffer of pH 3.5.—The assemblage of organisms utilized to study the antibacterial action of hydrogen ions comprised a group of Gram-positive and Gram-negative bacteria commonly encountered as pathogens on skin and mucous membranes. The concentration of this cation to inhibit the growth of the test organisms was established from pH measurements of inoculated broth containing varying quantities of the acid. The minimum concentration of hydrogen ions, expressed as pH, to completely inhibit bacterial growth is presented in Table III. It is noteworthy to observe that growth inhibition of bacteria generally occurred in a rather narrow range of hydrogen-ion concentration, corresponding to pH 4.1 to 4.9. In addition, one organism of the test group, *Corynebacterium diphtheriae*, was especially sensitive to the action of hydrogen ions, as shown by inhibition of growth occurring at pH 6.6. On the basis of these findings, a conclusion appears warranted regarding the reliance of hydrogen ions in its inhibitory effect on bacterial growth and the probable advantage gained from clinical use of a buffer of pH 3.5 to combat bacterial infections when applicable.

The nature of the antibacterial action caused by hydrogen ions at the concentration level of the peptone-gluconic acid buffer used was studied by the F. D. A. immersion test technique (11). In general, a bacteriostatic action was observed, although for *C. diphtheriae* the buffer product proved decidedly bactericidal, and for *Streptococcus pyogenes*, bactericidal to a less marked degree. Table III includes these data.

TABLE III.—pH LEVEL FOR INHIBITION OF BACTERIAL GROWTH AND ANTIBACTERIAL ACTION OF PEPTONE-GLUCONIC ACID BUFFER OF pH 3.5

Organism	Gram's Stain	pH for Inhibition of Bacterial Growth	Antibacterial Action of Buffer of pH 3.5
Escherichia coli	−	4.65	Bacteriostatic
Proteus vulgaris	−	4.10	Bacteriostatic
Klebsiella pneumoniae	−	4.65	Bacteriostatic
Aerobacter aerogenes	−	4.10	Bacteriostatic
Pseudomonas aeruginosa	−	4.25	Bacteriostatic
Neisseria catarrhalis	−	4.65	Bacteriostatic
Staphylococcus aureus	+	4.70	Bacteriostatic
Staphylococcus albus	+	4.70	Bacteriostatic
Streptococcus pyogenes	+	4.65	Bactericidal[a]
Corynebacterium diphtheriae	+	6.60	Bactericidal[b]

[a] Bactericidal in thirty-minutes immersion test.
[b] Bactericidal in one-minute immersion test.

Inhibition of bacterial growth appears to be immediate once contact is made between organism and hydrogen ions. An examination of *E. coli* showed complete and prompt cessation of motility on adding the buffer product to a suspension of the bacteria. A similar immediate response was obtained with *Trichomonas vaginalis* organisms.

Hydrogen ions exhibited a rather general action on all bacteria, regardless of Gram reaction. This is an advantage over the antibiotics and sulfonamides which vary in their effectiveness against Gram-positive and Gram-negative organisms because of their specificity.

The pH causing inhibition of bacterial growth was determined by varying the amount of gluconic acid added to inoculated culture broth, and the pH was read after forty-eight hours incubation at 37°, using a Beckman pH meter with glass electrode. Antibacterial action of peptone-gluconic acid buffer of pH 3.5, containing 1% of the base constituent, was observed by employing the standard F. D. A. immersion test. The bacteria used in these tests were obtained from a twenty-four-hour stock culture in nutrient broth.

The importance of buffer action for maintaining the concentration of hydrogen ions at the required level to achieve sustained antibacterial effect was demonstrated by comparison of the gluconic acid buffer of pH 3.5 and unbuffered gluconic and hydrochloric acids of the same pH, on the growth of *Staphylococcus aureus* and *E. coli* by the agar cup-plate method. Inhibition of growth of these two organisms did not result around the cups of the agar plate filled with the unbuffered acids, presumably because of neutralization of hydrogen ions as these

[1] Calcium salt of gluconic acid has been extensively employed in medicine in recent years for calcium therapy. It is administered orally or parenterally in comparatively large doses, and its pharmacological action and toxicity is due to calcium ions.

[2] It was found, aside from the hydrogen-ion concentration, that undissociated gluconic acid, its lactone, and gluconate ions are all ineffective against bacteria. These findings are based upon the observations that sodium gluconate, added up to 6% concentration to nutrient broth, failed to inhibit the growth of *Escherichia coli*. Furthermore, the growth of *E. coli* was inhibited at pH 4.6 ± 0.05 when the hydrogen-ion concentration of nutrient broth medium was adjusted with hydrochloric acid. Gluconic acid added to the medium produced inhibition of growth at the same pH.

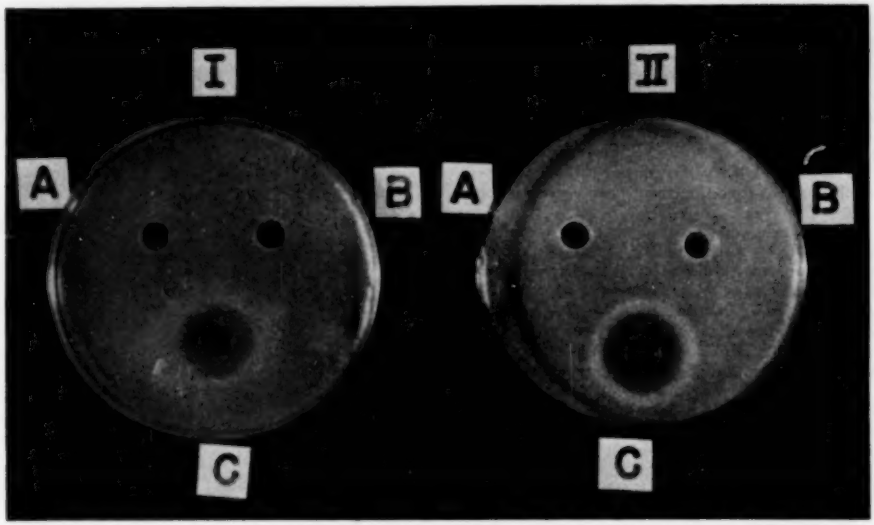

Fig. 4.—Demonstration of antibacterial action of peptone-gluconic acid buffer of *p*H 3.5 by agar cup-plate method, exhibiting effect on growth of (*I*) *Staphylococcus aureus* and (*II*) *Escherichia coli*. Agar cup plates filled with (*A*) hydrochloric acid solution of *p*H 3.5, (*B*) gluconic acid solution of *p*H 3.5, and (*C*) peptone-gluconic acid buffer of *p*H 3.5, containing 5% of base constituent. Incubation period was for forty-eight hours at 37°. Note zone of inhibition and outside ring of stimulation in both agar plates.

ions diffused through the agar medium. In sharp contrast, the buffered acid produced marked zones of inhibition of bacterial growth. Figure 4 displays the results obtained by the agar plate experiment.

The antibacterial potency of hydrogen ions was determined and compared with a number of established antibacterial products, including antibiotics and organic synthetics. The potency of these agents was evaluated by ascertaining the minimum concentration necessary to arrest growth of the test organisms, *Staphylococcus aureus*, *Staphylococcus albus*, *E. coli*, and *S. pyogenes*. These test results on potency are shown in Table IV.

A concentration of 0.022 μg./ml. or less of hydrogen ions produced inhibition of growth for all test organisms. Greatest potency was shown against *C. diphtheriae* at a concentration of hydrogen ions of 2.5×10^{-4} μg./ml. These levels represent higher potency for hydrogen ions than that shown by the other antibacterial agents used in the comparison, on a weight basis. However, considered on a formula-weight basis, the order of potency shown by hydrogen ions would be altered.

DISCUSSION

The essential feature for the successful application of hydrogen ions for antisepsis is the establishment of the ion level at a range adequate to generally inhibit the growth of bacteria, yet base enough

TABLE IV.—COMPARISON OF ANTIBACTERIAL POTENCY OF HYDROGEN IONS WITH OTHER ANTIBACTERIAL AGENTS

Antibacterial Agent	μg./Ml. to Effect Complete Inhibition of Growth of Organism in Inoculated Broth after 24 Hr. Incubation at 37°			
	S. aureus	*S. albus*[a]	*E. coli*	*S. pyogenes*
Hydrogen ions[b]	0.020	0.020	0.022	0.022
Dihydrostreptomycin[c]	...	6.25	...	...
Aureomycin[c, e]	...	1.5	...	...
Penicillin[c, e]	0.05	3.0	38	0.11
Chloromycetin[c]	...	15.6	...	...
Gramicidin[d]	100	...	...	10
Merthiolate[d]	50	...	...	50
Zephiran[d]	20	...	...	8
Phemerol[d]	16	...	...	10
Sulfathiazole[d]	2,500	...	...	2,500

[a] Hemolytic-type organism.
[b] Hydrogen-ion concentration determined from *p*H data.
[c] Some decomposition of antibiotic occurred during twenty-four-hour incubation test.
[d] From data reported by Herrell, W. E., and Heilman, D., *Am. J. Med. Sci.*, **206**, 221(1943). Inhibition was not complete with sulfathiazole.
[e] Concentration of dihydrostreptomycin given as the sulfate, aureomycin as the hydrochloride, and penicillin expressed as penicillin-G sodium, 1 mg. being equivalent to 1,667 antibiotic units.

to prevent ion toxicity against the tissues. The value of *p*H 3.5 was therefore instituted between the limits of *p*H 4.1, which was found requisite for inhibition of bacterial growth, and *p*H 2.5, approximately the minimum concentration of hydrogen ions observed to exhibit apparent toxicity to conjunctiva, skin, and subcutaneous tissue.

The findings of the hydrogen-ion toxicity studies are in agreement with the observations of Freidenwald, *et al.* (12), who found that irreversible changes in the cornea of the eye occur at *p*H 2.5 and below, as well as *p*H 11.5 and above. Any chemical injury initiated to conjunctiva or other no less sensitive tissues at *p*H 3.5 should therefore be entirely reversible. Though relative safety is assured by the results of the toxicity studies in using a preparation of *p*H 3.5, bland properties should not be ascribed to this concentration of hydrogen ions. Rather, a very mild degree of irritation appears to be present in view of the brief vasodilatation observed in the human eye experiment.

The effectiveness of the buffer product of *p*H 3.5 to inhibit bacterial growth appears to be quite general on the basis of the studies *in vitro*. A few exceptions occur with such organisms as *Lactobacilli*, *Streptococcus lactis*, and *Thiobacillus thiooxidans* (13), but these are not encountered as pathogens. The effect of the buffer on *Mycobacterium tuberculosis* was not determined.

The toxicity of hydrogen ions to bacteria involves various phenomena such as alteration of surface charge of bacteria, changes in the bacterial proteins, and limitation of enzyme activities. The rate of penetration of hydrogen ions per unit mass of bacterial cell should be an important factor, determining the degree of toxicity. Compared to bacteria, tissue cells present a much smaller surface area per unit mass for penetration of the ions. These differences in size between bacteria and tissue cells constitute a very likely explanation, at least in part, for the differences observed in the toxicity of hydrogen ions toward bacteria and animal tissues.

The effectiveness of the buffer against bacteria does not necessarily extend to other microorganisms. The fungi are not inhibited at *p*H 3.5.

The utilization of a buffer system to control *p*H serves to effect antibacterial action at a lower concentration of hydrogen ions than would otherwise be possible by an unbuffered system. Greater quantities of the unbuffered acid solution would be required to maintain an antiseptic environment, or a solution of acid with lower *p*H would be necessary to yield equivalent antibacterial response.

SUMMARY

1. Conditions necessary for application of controlled hydrogen-ion concentration for antisepsis have been established.

2. A buffer product of *p*H 3.5 produced a hydrogen-ion concentration adequate to inhibit growth of bacteria, and was without apparent harmful local effect on body tissues.

3. Growth of pathogenic bacteria was found to be inhibited at *p*H 4.1 or higher.

4. Hydrogen ions were much more effective against bacteria if the acid preparation was buffered.

5. For extended buffer action, a weak base should be used for the base constituent in the buffer, in addition to an acid having an ionization constant, Ka, of 1×10^{-4} found from theoretical considerations with the log (Salt)/(Acid) ratio set at -0.5 rather than 0.0.

6. The antibacterial potency of hydrogen ions was observed to be high, exceeding that of other antibacterial agents, including antibiotics, considered on a weight basis.

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2-Phenyl-4-Thiazolidone Derivatives as Local Anesthetics*

By F. P. LUDUENA and JAMES O. HOPPE†

Laboratory tests with some 2-phenyl-4-thiazolidone derivatives recently synthesized by Surrey (1) have disclosed significant local anesthetic activity. On the basis of these preliminary results, more compounds were synthesized and tested. Toxicity and local tissue irritation were determined for the most active compounds in the series. The various results obtained are described in this paper.

PRELIMINARY SCREENING by Tatum's external canthus test of the 2-phenyl-3-diethylaminoethyl-4-thiazolidone and the 4-aminophenyl, the 4-methoxyphenyl, the 3,4-dimethoxyphenyl, the 3,4,5-trimethoxyphenyl, and the 3,4-methylenedioxyphenyl analogs showed that the latter was comparatively the most active as a local anesthetic.

A series of 3,4-methylenedioxyphenyl-3-alkyl- or arylaminoalkyl-4-thiazolidone compounds were tested by the sciatic nerve block method. Their toxicity was determined by intravenous injection in mice. The local anesthetic activity and the toxicity appear to increase with the number of carbons of the amino substituent.

Two compounds, the 2-(3,4-methylenedioxyphenyl)-3-(3-isobutylaminopropyl)-4-thiazolidone (WIN 2661) and the 3-(2-cyclohexylaminoethyl) analog (WIN 2663) were also tested by intraspinal injection in rabbits, and by intradermal injection in comparison with procaine in man. Local irritation was determined by intradermal injection (trypan blue test) and by eye instillation in rabbits.

These two compounds were found to possess a higher anesthetic potency than procaine by the three methods used. They were correspondingly more toxic and somewhat more irritating.

METHODS

Tests for local anesthetic activity were made on each of the compounds identified in Tables I and II in solution as the hydrochloride salt. These methods of testing are described briefly in the following paragraphs. In all the methods used, the local anesthetic compound was dissolved in saline solution and the *p*H adjusted to a value between 5.5 and 6.5.

External Canthus.—Injection of 0.5 cc. subcutaneously under the external canthus of the rabbit's eye, as described by Tatum (2), was used for most of the preliminary tests. With this method, abolition of the wink reflex elicited by touching the eyelashes of the external side of the upper lid was taken as a sign of anesthesia.

Sciatic Nerve Block in Guinea Pigs.—The anesthetic solution (0.2 cc.) was injected close to the sciatic nerve according to the technique described by Shackell (3), with the difference that the animals were not tied on a board, but simply held with the left hand during the injection. Sensory block was determined by pricking the posterior side of the thigh with a needle. Pinpricks provoke squeals in the normal animal.

Of the several symptoms which Loomis and Spielmeyer (4) have described as following sciatic motor block in the guinea pig, the abnormal position of the leg on the injected side was used routinely as an indication of the presence of motor block. When the experimental animal is placed on a flat surface, the injected leg remains at right angle with the flank of the animal when the block is complete, regaining gradually the normal position as the motor block diminishes. The effect of the compounds was determined by the presence or absence and the duration of motor block, which lasts longer than the sensory block. The minimum of three animals per dose level was used. The values for WIN 2661 and WIN 2663 were obtained with 16 animals per dose level.

Corneal Anesthesia in Rabbits.—By pulling the lower lid upward and away from the eyeball, a sac is formed which then was filled with anesthetic solution. Usually 0.25 to 0.5 cc. is required to fill this sac. The solution covers all the corneal area for one minute, after which the lid is released and the excess solution allowed to escape. There is no rinsing of the cornea, the excess fluid draining away by natural means.

The wink reflex is provoked by applying gently on the cornea the blunt point of an L-shaped glass rod. The pressure must be sufficient to produce a slight depression of the cornea. The treated eyes were tested every five minutes.

Spinal Anesthesia.—Two compounds were tested by intraspinal injection in rabbits according to the method of Bieter, *et al.* (5). The amount injected was 0.02 cc. of the solution/cm. of spinal length. However, in our experiments, the area anesthetized was determined by pricking the skin with a needle. In the normal animal a pinprick on the back provokes a reflex skin twitch; on the thigh or foot, withdrawal of the leg. Pricking of the leg, in the presence of motor paralysis without sensory anesthesia, usually elicits other motor reactions in the upper part of the body. It is not easy to determine accurately the duration of anesthesia, as this wears off gradually.

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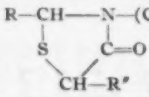
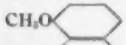
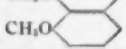
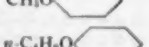
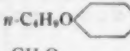
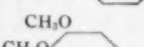
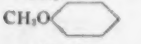
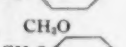
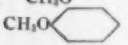
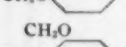
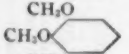
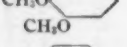
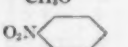
† The technical assistance of Miss Bernice Dertinger, Mrs. Ellen Miller, Mr. D. K. Seppelin, and Miss Jacqueline Sherndal is gratefully acknowledged.

TABLE I.—2-PHENYL-THIAZOLIDONE DERIVATIVES—LOCAL ANESTHESIA AND TOXICITY

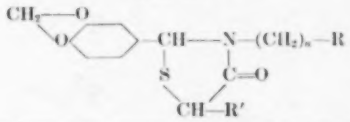


Compound		Structure				Average Duration of Anesthesia (Min.)				Acute Toxicity i.v. Mice, Mg./Kg.
	WIN No.	n	R	R′	R″	Solution, %	External Canthus Test	Sciatic Nerve Block	Corneal Instillation	
1	WIN 2131	2		$N(CH_3)_2$	H	0.5	5	..	..	...
2	WIN 2095	3		$N(C_2H_5)_2$	H	0.5	2	..	0	60[a]
3	WIN 3075	3		NH—*n*-C_4H_9	H	0.4	..	20	..	34 ± 1
4	WIN 3077	3		NH—*n*-C_4H_9	C_2H_5	0.2 0.1	.. ..	41 11	.. ..	30 ± 1.5 ...
5	WIN 2530	2		$N(C_2H_5)_2$	H	1.0	10	..	..	...
6	WIN 2776	3		NH	H	0.4 0.2	.. ..	54 35	.. ..	20 ± 1 ...
7	WIN 3151	3		NH—*n*-C_3H_7	H	0.4 0.2	.. ..	103+ 19	.. ..	40 ± 4 ...
8	WIN 2501	2		$NH(C_2H_5)_2$	H	1.0	11	..	..	120 ± 2
9	WIN 3094	3		NH—*n*-C_4H_9	H	1.0 0.5	.. ..	.. 19	25	63 ± 3 ...
10	WIN 2503	2		$N(C_2H_5)_2$	H	1.0	15	..	..	...
11	WIN 2502	2		$N(C_2H_5)_2$	H	0.5	..	6	..	56
12	WIN 3107	3		NH—*n*-C_4H_9	H	1.0 0.5	.. ..	38 20	.. ..	12
13	WIN 2531	2		$N(C_2H_5)_2$	H	1.0	5	..	..	...
14	WIN 3131	3		NH—*n*-C_4H_9	H	1.0 0.5	.. ..	53 14	20 ..	24 ± 1 ...
15	WIN 2126	2		$N(n\text{-}C_4H_9)_2$	H	0.5 0.1	86 18	.. ..	0 ..	32 ± 2 ...
16	WIN 3050	3		NH—*n*-C_4H_9	H	1.0	65+	..	..	5
17	WIN 2195	2		$N(C_2H_5)_2$	H	0.5 0.25	28 17	.. ..	.. ..	56 ± 3 ...

[a] Single value without standard errors = approximate value.

The best end point for spinal anesthesia in the rabbit is given by the reappearance of a reaction designated here as the "urethral reflex." This is provoked by the sudden injection of cold water into the urethra by means of a thin rubber catheter and syringe. The water spills out around the catheter which is introduced about 1 cm. into the urethra. This sudden dilatation reflexly provoked a contraction of the *sphincter ani externus* and apparently also of the urethral sphincter. In the normal animal, weaker stimulus, like touching the meatus or the introduction of the catheter, may elicit the reflex. The reappearance of this reflex is sudden and almost as conspicuous as that of the wink reflex.

TABLE II.—3,4-METHYLENEDIOXY-2-PHENYL-4-THIAZOLIDONE DERIVATIVES—LOCAL ANESTHESIA AND TOXICITY



Compound WIN No.	n	R	R′	Solution, %	Average Duration of Anesthesia, Min. External Canthus Tests	Sciatic Nerve Block	Corneal Instillation	Acute Toxicity i.v. Mice, Mg./Kg.
18 WIN 2125	2	$N(C_2H_5)_2$	H	1.0	64	26	27	68 ± 4
				0.5	23	..	..	...
				0.25	16	..	..	...
19 WIN 2761	2	$NH—C_2H_4OH$	H	1.0	9	..	..	180
20 WIN 2777	2	$N(n\text{-}C_4H_9)_2$	H	0.4	..	67	..	22 ± 1
				0.2	..	42	..	...
				0.1	..	19	..	...
21 WIN 2663	2	NH–(S)	H	1.0	..	..	19	30 ± 1
				0.4	..	47	..	...
				0.2	..	38	..	...
				0.1	..	14	..	...
22 WIN 2687	3	$N(C_2H_5)_2$	H	1.0	..	38	..	63 ± 5
				0.25	..	4	..	...
23 WIN 3039	3	$NH—C_2H_4OH$	H	1.0	..	16	..	85
24 WIN 2772	3	$NH—n\text{-}C_3H_7$	H	1.0	..	49	..	50 ± 4
				0.5	..	12	..	...
25 WIN 2773	3	$NH—i\text{-}C_3H_7$	H	1.0	..	15	..	74 ± 4
				0.5	..	7	..	...
26 WIN 2774	3	$NH—n\text{-}C_4H_9$	H	0.4	..	20	..	32 ± 2
27 WIN 2775	3	NH–(S)	H	0.4	..	48	..	25 ± 2
				0.2	..	42	..	...
				0.1	..	18	..	...
28 WIN 2661	3	$NH—i\text{-}C_4H_9$	H	1.0	..	..	19	40 ± 1
				0.4	..	42	..	...
				0.2	..	16	..	...
				0.1	..	10	..	...
29 WIN 3028	3	$NH—n\text{-}C_5H_{11}$	H	0.4	..	62	..	18 ± 2
				0.1	..	19	..	...
30 WIN 3039	3	$NH—C_2H_4OH$	H	1.0	..	16	..	85
31 WIN 3133	3	$NH—n\text{-}C_6H_{13}$	H	0.2	..	38	..	25
				0.1	..	24	..	...
32 WIN 3132	3	$NH—n\text{-}C_8H_{17}$	H	0.1	..	46	..	7.2 ± 0.6
				0.05	..	21	..	...
33 WIN 3282	3	NH–(S)	H	0.2	..	22	..	36 ± 2
				0.1	..	4	..	...
34 WIN 3273	3	NH–(S)	C_2H_5	0.2	..	53	..	22 ± 1
				0.1	..	20	..	...
35 WIN 3078	3	$NH—CH_2$–(C6)	H	0.4	..	31	..	28 ± 1
				0.2	..	24	..	...
				0.1	..	5	..	...
36 WIN 3229	6	NH–(S)	H	0.5	..	69+	..	18
37 Procaine	...	...	...	2.0	..	32	..	62 ± 2
				1.0	..	23	..	...
				0.5	..	15	..	...
38 Cocaine	...	...	...	1.0	..	..	23	21 ± 1

The speed of penetration of the anesthetic can be estimated by the interval between the injection and the disappearance of the urethral reflex. After injection, the paraplegia may be evident as soon as the animal is lifted from the hammock, whereas the urethral reflex may disappear after one or two minutes.

Local Irritation.—To determine the local irritation produced by these compounds when applied on the rabbit's eye, the method and scoring system devised by Draize, *et al.* (6), were used. The degree of irritation produced by intradermal injection was determined by the trypan blue test. Trypan blue was used first by Hirschfelder (7) and Tainter and Hanzlik (8) to determine the part played by capillary permeability in paraphenylenediamine edema in experimental animals. Later Tainter, Throndson, and Lehman (9) devised a method to estimate the degree of irritation produced by solutions injected subcutaneously, based on the intensity of the local extravasation of trypan blue administered intravenously. A modification of this method, as described by Hoppe, Alexander, and Miller (10) was used in our investigation.

Intradermal Injection in Man.—Two compounds, WIN 2661 and WIN 2663, were tested in comparison with procaine by intradermal injection of 0.1 cc. of the sterile solutions on the volar side of the forearm. The area and duration of anesthesia were determined by pinpricks. Other local manifestations were also recorded.

Toxicity.—Acute toxicity determinations were made by intravenous injection into male Webster strain, albino mice weighing 22 ± 2 Gm. The compounds in aqueous solution were injected in dosages graduated at 0.1 logarithmic intervals into groups of 10 mice per dose level for the estimation of the LD_{50} ± its standard error by the method of Miller and Tainter (11) with results as shown in Tables I and II. The rate of injection was maintained at 1.0 cc. per minute. The volume of injection was restricted to 0.35 ± 0.15 cc. for each route of administration. The acute toxicity of two of the compounds in comparison with procaine hydrochloride was determined by subcutaneous injection in mice and intravenous injection in guinea pigs with results as shown in Table VI.

RESULTS

In a preliminary investigation, the influence of phenyl substitution was determined by comparing the local anesthetic activity, by the external canthus test, of compounds 5, 8, 10, 13, and 18 (see Table I). All these compounds have the procaine side chain. The most active compound was the 3,4-methylenedioxy-2-phenyl derivative and one of the least active, the 4-aminophenyl analog.

Based on these results, more 3,4-methyienedioxy 2-phenyl-4-thiazolidones were synthesized and tested. The results with all the 4-thiazolidone analogs are summarized in Tables I and II.

The two compounds with the 4-chloro-phenyl substitution produced anesthesia of long duration. Compound 15 had no topical anesthetic activity, but produced marked corneal irritation at 1% concentration.

In the series of 3,4-methylene-dioxyphenyl secondary amines, the activity increased in direct proportion to the length of the side chain from the *n*-propylamino- to the *n*-octylamino-derivative. This last compound has very poor water solubility. The local irritant properties also appeared to increase with the number of carbons of the alkyl- or cycloalkylamino substituent.

In general, a very close correlation between local anesthetic activity and toxicity of these compounds was found.

Local anesthetic activity is lost or greatly reduced by oxidation of the sulfur in the thiazolidone ring. The following compounds were found inactive when tested by the sciatic nerve block method at 1% concentration: 3-(3-butylaminopropyl) -2-phenyl-4-thiazolidone-1-dioxide (WIN 3076), 3-(2-diethylaminoethyl)-2-(4-methoxyphenyl)-4-thiazolidone - 1-dioxide (WIN 2709) and 3-(2-diethylaminoethyl)-2-(3,4-methylenedioxyphenyl)-4-thiazolidone - 1 - dioxide (WIN 2662). Very slight anesthetic activity was observed with another compound in this series, 3-(3-amylaminopropyl)-2-(4 -chlorophenyl) - 4 - thiazolidone-1-dioxide (WIN 3093).

Additional tests were run with compounds 21 and 28 (WIN 2661 and WIN 2663) in comparison with procaine, as follows.

Spinal Anesthesia in Rabbits.—The results are summarized in Table III. No acute deaths were observed with the highest concentration of the three compounds used. One difference observed between the two WIN compounds and procaine was that with the former the disappearance of the urethral reflex was not observed for one or two minutes after the injection.

TABLE III.—SPINAL ANESTHESIA IN RABBITS

Compound	Concn., %	No. of Rabbits	Duration of Anesthesia Urethra[a] Av.	Range
WIN 2661	0.5	5	33	30–33
	1.0	7	57	40–100
	2.0	5	84	74–90
	4.0	5	175	70–220
WIN 2663	0.5	5	34	20–46
	1.0	5	36	30–42
	2.0	5	61	50–70
Procaine HCl	1.0	4	20	14–28
	2.0	5	36	29–51
	4.0	5	44	40–50

[a] Minutes.

Intradermal Injection in Man.—WIN 2661 produced anesthesia of longer duration than WIN 2663, and both were more active than procaine (Table IV). The addition of epinephrine hydrochloride to the solutions of WIN 2663 greatly increased the duration of the anesthetic effect. No subjective or objective signs of local irritation were observed with these compounds.

Local Irritation.—By the trypan blue test, WIN 2661 was less irritating than WIN 2663 and more than procaine (Table V). These compounds, in solutions of 0.5%, produced no stinging or other signs of irritation when injected intradermally in man.

Topically, the degree of irritation observed in the eye after application was of a lesser degree than when injected intradermally in the same animal. This was generally observed with all the local anesthetics tested.

TABLE IV.—INTRACUTANEOUS ANESTHESIA PRODUCED BY WIN 2663 ON HUMAN SUBJECTS

Compound	Concn., %	No. Positive/ No. Tested	Duration in Min. Av.	Range
WIN 2663	0.1	5/5	20	12–25
	0.25	5/5	27	17–45
	0.5	5/5	41	17–65
WIN 2661	0.1	5/5	30	18–45
	0.25	5/5	41	35–55
	0.5	5/5	52	35–65
Procaine HCl	0.5	10/10	14	7–25
	1.0	5/5	18	12–25
WIN 2663 + epinephrine 1:50,000	0.1	5/5	25	15–35
	0.25	5/5	127+	100+–170+
Procaine HCl + epinephrine 1:50,000	0.5	5/5	84+	55–125+
	1.0	5/5	106+	65–155+

Toxicity.—In general, the toxicity was directly related to the local anesthetic activity. The LD_{50} values varied from 12 to 74 mg./Kg., the majority being more toxic than procaine. The toxicity of the compounds with very low anesthetic activity was not determined; higher LD_{50} values may have been obtained in those cases.

WIN 2663 was somewhat more toxic than WIN 2661, which in turn was more toxic than procaine as determined by intravenous and subcutaneous injection in mice, and by intravenous injection in guinea pigs (Table VI).

Effects on Anesthetized Dogs.—WIN 2663 and WIN 2661 had negligible effect when injected intravenously in doses of 1 mg./Kg. in barbitalized dogs. Doses of 2 to 5 mg./Kg. produced moderate falls of pressure. Very little or no effect on heart rate was produced by these doses. The respiratory rate was moderately increased. Tonus of the small intestine was decreased.

Procaine and tetracaine were tested in the same manner. They produced essentially the same effect, but procaine appeared to be weaker than WIN 2663, whereas the effect of tetracaine was greater and more prolonged.

TABLE VI.—TOXICITY

Compound	Subcutaneous Injection in Mice, Mg./Kg. ± Standard Error	Intravenous Injection, Mg./Kg. ± Standard Error	
		Mice	Guinea Pigs
WIN 2663	190 ± 10	30 ± 1	18 ± 1
WIN 2661	320 ± 30	40 ± 1	20 ± 1
Procaine HCl	660 ± 60	62 ± 2	60 ± 3

DISCUSSION

The results presented above show that substituted 2-phenyl-3-alkylaminoalkyl-4-thiazolidone compounds possess local anesthetic activity. The 3,4-methylenedioxy-2-phenyl-4-thiazolidone structure resembled the 4-aminobenzoate moiety in the fact that the attachment of anesthesiophoric side chains resulted in compounds in both series with comparable local anesthetic activity.

Several compounds in the 3,4-methylenedioxy group are more active than procaine as local anesthetics, as judged by the comparison of their duration/concentration curves. On this basis, WIN 2661 and WIN 2663 are two to four times as active as procaine.

However, by intraspinal injection in rabbits, WIN 2661 and WIN 2663 are only twice as active as procaine, and evidently slower in inducing anesthesia.

It appears that the diffusion into and out of the tissues, including nervous tissue, from the point of injection is more rapid with procaine than with WIN 2661 and WIN 2663, as shown by the slowness of induction of spinal anesthesia and the relatively long duration of nerve block with the higher doses of these compounds in comparison with those of procaine.

CONCLUSIONS

1. Most of the members of a series of 3-(alkyl, dialkyl and cyclo-alkylaminoalkyl)-2-phenyl-4-thiazolidone derivatives were found to possess local anesthetic activity when tested by the external canthus and the sciatic nerve block methods in rabbits and guinea pigs, respectively.

2. 1-Dioxide-4-thiazolidone analogs of some of the compounds in the above series showed very poor or no local anesthetic activity.

TABLE V.—LOCAL TISSUE IRRITATION IN RABBITS

Compound	Concn., %	Intradermal Irritation (Trypan Blue Test) No. of Tests	Intradermal Irritation (Trypan Blue Test) Maximum Average Irritation Score	Intradermal Irritation (Trypan Blue Test) Adjective Rating	Mucous Membrane Irritation (Rabbit Eye Test) No. of Tests	Mucous Membrane Irritation (Rabbit Eye Test) Maximum Average Irritation Score	Mucous Membrane Irritation (Rabbit Eye Test) Adjective Rating
WIN 2661	1	2	3	Mild	2	0	None
	2	2	4	Moderate	2	1	Mild
WIN 2663	1	2	6	Moderate	2	0	None
	2	2	12	Marked	2	3	Mild
	4	2	16	Marked	2	6	Moderate
Procaine HCl	5	5	2	Mild	..	...	...
	10	5	16	Marked	..	...	...
	20	5	16	Marked	..	...	...
Cocaine HCl	2	..	..	...	5	0	None
	4	..	..	...	5	0.8	Mild
	8	..	..	...	5	0.8	Mild
	16	..	..	...	5	2.8	Mild

3. WIN 2661 (3,4-methylenedioxy-2-phenyl-3-isobutylaminopropyl-4-thiazolidone) and the 2-cyclohexylaminoethyl analog were two to four times as active as procaine in producing sciatic nerve block in guinea pigs.

4. By intraspinal injection in rabbits, both WIN 2661 and WIN 2663 were about twice as active as procaine. They were less active than cocaine when applied topically on the cornea.

5. By the intradermal wheal test in man, both WIN 2661 and WIN 2663 were clearly more active than procaine and their effect was increased by the addition of epinephrine 1:50,000 to the solutions.

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Solubility of Phenobarbital in Alcohol-Glycerin-Water Systems*

By GEORGE M. KRAUSE† and JOHN M. CROSS‡

The solubility of phenobarbital in alcohol, water, glycerin, and combinations of these solvents has been determined and is reported. It has been found that the solubility of phenobarbital in hydroalcoholic solutions reaches a maximum at 90 per cent alcohol. The solubility of phenobarbital in alcohol is increased by the addition of glycerin, although glycerin and glycerin-water solutions are poor solvents for this compound. Tables and charts are included for use in determining quickly the volume of alcohol and/or glycerin required to dissolve a given weight of phenobarbital.

ONE of the problems facing the pharmacist today is the determination of the quantity of alcohol necessary to dissolve and keep in solution a definite weight of phenobarbital. Since many liquid preparations and prescriptions contain phenobarbital as one of the ingredients, the problem involved is of practical interest.

Phenobarbital is relatively insoluble in aqueous or low alcoholic solutions, whereas the sodium salt is soluble in both alcohol and water. This advantage is negated by the fact that the sodium salt is not stable in solution, breaking down into phenylethylacetylurea, an inactive decomposition product (1).

Since glycerin is often present in vehicles and other liquid preparations of phenobarbital, it was felt that the effect of glycerin on the solubility of phenobarbital in hydroalcoholic solutions should be studied. A search of the literature did not disclose information relative to the problem.

Therefore it was decided to determine the solubility of phenobarbital in various concentrations of alcohol and water (2), glycerin and water, and alcohol-glycerin-water mixtures, and from this information to prepare a table showing the solubility of phenobarbital in such mixtures.

Since the usual method of determining solubility, i.e., evaporation of the solvent, is not practicable where glycerin is a solvent, a more suitable method of analysis was necessary.

The potentiometric method of Waters, Berg, and Lachman (3) offered advantages in that it was not necessary to use indicators and to rely on color change to detect the end point of the titration.

A somewhat similar method was employed by Mattocks and Voshall (4). The instrument employed was a Fisher Senior Titrimeter, using a silver electrode and a silver-silver chloride electrode, with 0.1 *N* silver nitrate as the titrant.

After studying these methods, it was decided to conduct a potentiometric analysis for the phenobarbital based on the method of Waters, *et al.* (3), as modified by Bartilucci and Discher (5, 6), using 0.1 *N* sodium hydroxide as the titrant.

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The results of 10 titrations indicate the method is accurate to within about 0.5 per cent.

TABLE I.—RESULTS OF TEN TITRATIONS FOR DETERMINATION OF ACCURACY OF POTENTIOMETRIC METHOD

Sample No.	Wt. of Phenobarbital Sample	Wt. of Phenobarbital Sample Determined by Assay	Theoretical Amount, %	Deviation per Sample, %
1	0.3868	0.3871	100.08	+0.08
2	0.2645	0.2644	99.99	−0.01
3	0.2431	0.2430	99.98	−0.02
4	0.6742	0.6746	100.07	+0.07
5	0.3919	0.3918	99.98	−0.02
6	0.3575	0.3584	100.27	+0.27
7	0.5514	0.5506	99.87	−0.13
8	0.4739	0.4764	100.53	+0.53
9	0.6146	0.6178	100.53	+0.53
10	0.4444	0.4464	100.47	+0.47
		Av.	100.17	

EXPERIMENTAL

Preparation of Solutions.—The solvents and solvent mixtures used were as follows:

(*a*) Water.
(*b*) Alcohol, U. S. P.
(*c*) Glycerin, U. S. P.
(*d*) Absolute alcohol.
(*e*) Distilled water and alcohol.
(*f*) Distilled water and glycerin.
(*g*) Absolute alcohol and glycerin.
(*h*) Distilled water, alcohol, and glycerin.

In all cases solutions were made up to represent per cent volume. Where glycerin was used, it was weighed and calculated to represent an equivalent volume. Throughout this paper the term "per cent alcohol" indicates per cent by volume of C_2H_5OH.

To the proper solvent or solvent combination placed in a screw-capped glass vial, phenobarbital was added slightly in excess of the amount required for saturation. The tops of the vials were sealed by dipping them into paraffin. The vials were then placed in a constant-temperature bath at 25 ± 0.1° and rotated for forty-eight hours. At the end of this period, two 15-ml. samples of the clear liquid were withdrawn and analyzed according to the procedure outlined.

The data are shown in the following figures and tables:

Figure 1.—Solubility of phenobarbital in absolute alcohol-glycerin mixtures.

Figure 2.—Solubility of phenobarbital in (*a*) glycerin-water, (*b*) alcohol-water, and (*c*) absolute alcohol-glycerin solutions.

Figure 3.—Solubility of phenobarbital in alcohol-glycerin-water mixtures.

Table I.—Results of analysis of 10 known samples of phenobarbital by method described.

Table II.—Chart showing per cent of alcohol (U. S. P.) in aqueous solution required to dissolve a given number of grains of phenobarbital per fluid ounce. The results shown compare favorably with the findings of Leuallen (2).

Table III.—Table indicating the per cent solubility of phenobarbital in various mixtures of the solvents listed.

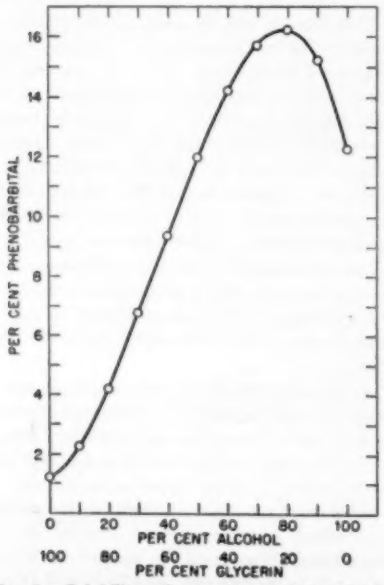

Fig. 1.—Solubility of phenobarbital in absolute alcohol-glycerin mixtures.

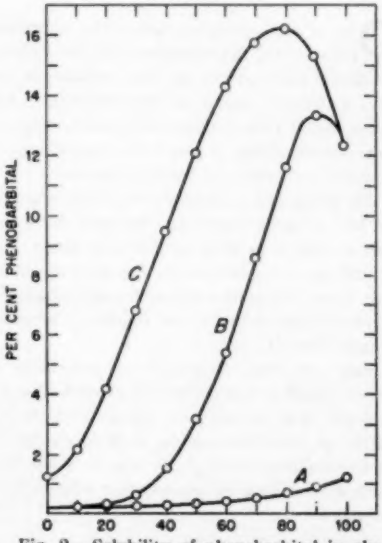

Fig. 2.—Solubility of phenobarbital in glycerin, alcohol, and absolute alcohol-glycerin. Abscissa indicates percentage of: *A*, glycerin in water; *B*, alcohol in water; *C*, absolute alcohol in glycerin.

TABLE III.—SOLUBILITY OF PHENOBARBITAL IN ALCOHOL-GLYCERIN-WATER SYSTEMS AT 25 ± 0.1°

(1) ALCOHOL	GLYCERIN 0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
0%	0.12	0.19	0.20	0.21	0.23	0.28	0.37	0.48	0.66	0.82	1.16
10%	0.19	0.22	0.30	0.37	0.50	0.64	0.84	1.14	1.59	2.25	
20%	0.30	0.42	0.57	0.83	1.13	1.54	2.11	2.77	4.13		
30%	0.64	0.93	1.35	1.89	2.67	3.45	4.45	6.79			
40%	1.46	2.16	2.87	4.09	5.37	6.83	9.40				
50%	3.21	4.26	5.57	7.18	9.29	12.01					
60%	5.33	7.47	9.19	11.23	14.25						
70%	8.53	10.73	12.75	15.74							
80%	11.56	13.58	16.27								
90%	13.38	15.30									
100%	12.30										

NOTE: Where the sum of the percentages of alcohol and glycerin mixtures does not equal 100, the difference is water. Where the sum equals 100, no water is present in such mixtures.

TABLE II.—APPROXIMATE PER CENT OF ALCOHOL REQUIRED TO DISSOLVE GRAINS OF PHENOBARBITAL PER FLUID OUNCE

Gr. of Phenobarbital/-Fl. Oz.	Equivalent to %	% by Volume of U.S.P. Alcohol Required for Solution
1	0.22	11.0
2	0.44	27.0
3	0.66	32.0
4	0.88	35.0
4.5	1.00	37.0
5	1.10	38.0
6	1.33	40.0
7	1.55	43.0
8	1.77	45.0
9	2.00	47.0
10	2.22	48.0
11	2.44	49.0
12	2.66	50.0
13	2.88	51.0
14	3.00	52.0
15	3.22	53.0

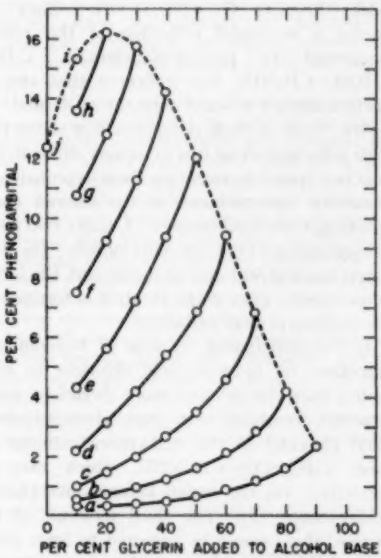

Fig. 3.—Solubility of phenobarbital in alcohol-glycerin-water mixtures, *a* 10%, *b* 20%, *c* 30%, *d* 40%, *e* 50%, *f* 60%, *g* 70%, *h* 80%, *i* 90%, *j* 100%.

SUMMARY

1. The solubility of phenobarbital at 25° in alcohol, water, glycerin, and combinations of these solvents has been determined.

2. A table has been prepared showing the solubility of phenobarbital in alcohol, glycerin, water, and combinations of these solvents.

3. It is interesting to note that the solubility of phenobarbital in hydroalcoholic solutions reaches a maximum at 90 per cent alcohol, and a decrease in solubility is noted in U. S. P. alcohol and absolute alcohol.

4. Glycerin and glycerin-water solutions are poor solvents for phenobarbital.

5. The solubility of phenobarbital in alcohol is enhanced by the addition of glycerin. The maximum solubility of phenobarbital in alcohol-glycerin mixtures is reached at a level of 80 per cent alcohol and 20 per cent glycerin.

6. A chart has been included for rapid determination of the quantity of U. S. P. alcohol required to dissolve a given quantity of phenobarbital.

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Amino Alcohols. XVIII. Reduction Studies on Arylglyoxylohydroxamyl Chlorides and Amides*

By JOSEPH P. LaROCCA†, WALTER H. HARTUNG‡, and NATHAN LEVIN§

A method for the preparation of arylglyoxylohydroxamamides from aliphatic and aromatic amines is described. The reaction has been applied to two aromatic amines, eight aliphatic amines, and to ammonia. Reduction studies of the compounds prepared are reported.

In 1942 Levin and Hartung (1) described the synthesis of arylglyoxylohydroxamyl chlorides, Ar—CO—C—(:NOH)—Cl. They reported a successful reduction of the phenyl compound to phenylethanolamine, C_6H_5—CHOH—CH_2NH_2, but indicated that the reduction did not proceed normally as desired. A further study of these reductions now shows that with palladium alone it is extremely difficult, and next to impossible, to reduce these hypothetically attractive intermediates to the desired corresponding arylethanolamines. Usually two moles of hydrogen are taken up quite rapidly; the third enters much slower and, as mentioned, the fourth enters rarely, even if the catalyst is fortified by the addition of more palladium.

It was anticipated, in view of Rosenmund's procedure for reducing acid chlorides to aldehydes, that the chlorine atom should be easily removed, resulting (for phenylglyoxylohydroxamyl chloride) in the well-known oximino ketone, C_6H_5—CO—CH:NOH, which may be converted, via the amino ketone, into phenylethanolamine (2). The third molecule of hydrogen, then, would be expected to form phenacylamine. In agreement with these expectations is the finding (1) that the partially reduced intermediate treated with alkali forms diphenylpyrazine; this suggests the presence of the amino ketone (2). The yield of crude diphenylpyrazine is good, but with a low melting point, and much of it is lost in purifying to maximum melting point. In any case, the ready absorption of two molecules of hydrogen and the further absorption of a third molecule would suggest that the reduction proceeds in stages, hence one would expect to isolate in good yields products which are intermediate between the hydroxamyl chloride and the arylalkanolamine. However, such is not the case. When the products were isolated at the several stages of hydrogenation, they were not homogeneous, and during the attempts to isolate pure materials, most of the product was lost. Furthermore, any products which could be isolated did not prove to be any of the intermediate stages which might be constructed, and they resisted further hydrogenation. This is even more surprising now that it has been found possible to obtain good yields of the corresponding amino alcohols from the starting materials.

Several other approaches were tried, namely: conversion of the hydroxamyl chloride into corresponding amides, Ar—CO—Cl(:NOH)—NRR, and a modification of the catalyst. This latter proved effective; several arylethanolamines have been prepared by this adaptation, described below, and it is believed that others will likewise be obtainable where the glyoxylohydroxamyl halide is available. Meanwhile the work via the amides had progressed to the point where these reduction studies also deserve mention.

Levin and Hartung (1) found that the arylglyoxylohydroxamyl chlorides behave as acid chlorides, and may be converted into amides as expected. For the present study a series of amides was prepared, for which the data are summarized in Table I. Several of these were then subjected to catalytic hydrogenation. Any one or a mixture of three possible reduction products might be expected (see compounds I, II, and III). Compound III was not expected since compounds in which two amino groups are attached to a single carbon are but little known; structurally they are ammono analogs of the hydrated form of an aldehyde, and would therefore be expected to be amenable to further reduction, forming in this instance I or II, or both. In the reduction of the simple amide C_6H_5—CO—C(:NOH)—NH_2 four moles of hydrogen was absorbed and the product was phenylethanolamine. But with the N-substituted amides, only three moles of hydrogen was taken up; analytical data for the hydrochloride of the reduction product from phenylglyoxylohydroxanilide indicate the formula $C_{14}H_{16}N_2O \cdot 2HCl$; analysis for the salt of

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For No. XVII of this series, see Simonoff, R., and Hartung, W. H., THIS JOURNAL, 35, 306(1946).
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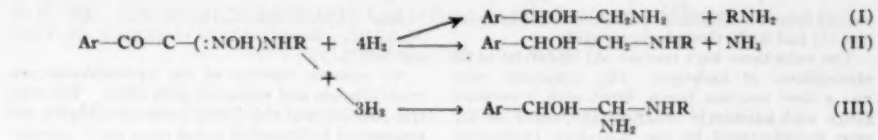

the product from the N-butyl analog suggests the formula $C_{13}H_{19}N_2O \cdot 2HCl$. Whether these have structures corresponding to III has not yet been established, and there is no explanation for the formation, apparently, of a dihydrochloride from the anilide and a monohydrochloride from the N-butyl intermediate.

As indicated, it is now found possible to reduce by catalytic methods the arylglyoxylohydroxamyl chlorides directly according to the equation $Ar—CO—C(:NOH)—Cl + 4H_2 \rightarrow Ar—CHOH—CH_2NH_2 \cdot HCl + H_2O$. The catalyst was modified by the addition of platinic oxide, and the hydrogenation was carried out at pressures varying from 10 to 20 atmospheres. Repeated experiments with phenylglyoxylohydroxamyl chloride showed that slightly more than the calculated (0.003 to 0.005 mole for 0.02 mole substrate) for complete reduction was absorbed in approximately ninety minutes. The product was phenylethanolamine. In a similar manner, the *p*-methyl- and the *p*-phenyl-phenylethanolamines were obtained from the corresponding hydroxamyl chlorides.

EXPERIMENTAL

The synthesis of arylglyoxylohydroxamyl chlorides has already been described. The amides were prepared as follows:

Separate solutions in anhydrous ether were prepared of two moles of amine and one mole of hydroxamyl chloride; the solution of the amine was added dropwise with vigorous stirring to the solution of the chloride. Reaction occurred instantaneously, as noted by the precipitation of the amine hydrochloride. The hydrochloride was filtered off, and the ethereal solution evaporated to dryness. The aromatic hydroxamamides were found to be solid. However, those formed with aliphatic amines were semisolid or oily in nature, and all attempts to crystallize them were unsuccessful. Phenylglyoxylohydroxamamide was prepared by bubbling ammonia gas (generated by heating concentrated ammonium hydroxide) through an ethereal solution of the chloride.

Hydrochlorides of the phenylglyoxylohydroxamamides were prepared by spraying an ethereal solution of the hydroxamamide with hydrogen chloride. The hydrochlorides were found to be white powders having sharp melting points and were used to identify the respective hydroxamamides. These salts were found to be fairly unstable and, on treatment with water, hydrolyzed readily to give the original hydroxamamide and hydrochloric acid. Table I summarizes the arylglyoxylohydroxamamides and their hydrochlorides, which were prepared by the procedures described.

The palladium chloride and platinic oxide were obtained from J. Bishop and Company. The palladium charcoal catalyst was prepared by essentially the same method as described by Hartung (3). To this catalyst, 0.150 Gm. of platinum oxide was added so that final composition of the catalyst

TABLE I.—SUMMARY OF ARYLGLYOXYLOHYDROXAMAMIDES

Ar·CO·C(:NOH)NHR							
Ar—	R—	Melting Point,[a]	Melting Point of Hydrochloride,[a]	Characteristics	Yield, %	Nitrogen, % Calcd.	Nitrogen, % Found[a]
Phenyl	Phenyl	142	123–124	Yellow flakes	62	11.60	11.69 11.57
Phenyl	β-Naphthyl	178–179	...	Yellow crystals	93	9.65	9.91 9.98
Phenyl	Hydrogen	129–131	...	Yellow prisms	56	17.08	17.23 17.15
Phenyl	Morpholino	98	...	Yellow flakes	2	11.96	12.20 12.08
Phenyl	Isopropyl	...	...	Semisolid	90		
Phenyl	*n*-Propyl	...	...	Semisolid	68		
Phenyl	Ethyl	...	...	Viscous oil	79		
Phenyl	Benzyl	...	...	Semisolid	82		
Phenyl	Methyl	...	132–133	Yellow oil	80	13.05[b]	13.53 13.79
Phenyl	*n*-Butyl	...	120–125	Viscous oil	85	10.99[b]	10.82 10.53
Phenyl	Heptyl	...	123–125	Yellow oil	70	9.39[b]	9.51 9.79
p-Methylphenyl	Phenyl	163–164[c]	...	Colorless needles	95		
p-Methylphenyl	Methyl	124	...	Colorless needles	75		

[a] Microanalyses by Oakwold Laboratories, Alexandria, Va.
[b] Assayed as hydrochloride.
[c] Agrees with value given in the literature.

was as follows: 10.0 Gm. palladium-Nuchar catalyst (3) and 0.150 Gm. platinum oxide.

The reductions were carried out under 10 to 20 atmospheres of hydrogen. The apparatus used was a steel reaction bomb, fitted with a pressure gauge and automatic shaking attachment of the type manufactured by the American Instrument Company.

In all the reductions performed the reaction solvent used was 95 ml. of 95% alcohol to which 5 ml. of concentrated hydrochloric acid had been added.[1]

The reduction products were isolated by removing the catalyst by filtration and evaporating the reduction mixture to dryness in a vacuum desiccator over P_2O_5.

Reduction of N-Phenylphenylglyoxylohydroxamamide.—From the reduction of 2.4 Gm. of N-phenylphenylglyoxylohydroxamamide, 2.2 Gm. of greenish crystals were obtained. The product was very hydroscopic and the highest melting point determined was 80–85°.

Anal.—Calcd. for $C_{14}H_{16}ON_2 \cdot 2HCl$: N, 9.35%. Found: N, 9.29% and 9.12%.

Reduction of N-Butylphenylglyoxylohydroxamamide.—From the reduction of 4 Gm. of N-butylphenylglyoxylohydroxamamide, 2.1 Gm. of white hydroscopic crystals, m. p. 140–145°, were obtained.

Anal.—Calcd. for $C_{12}H_{20}ON_2 \cdot HCl$: N, 11.49%. Found: N, 11.21% and 11.24%.

Reduction of Phenylglyoxylohydroxamamide.—Phenylethanolamine hydrochloride, (1.1 Gm.) m. p. 210–215° (decompd.) was obtained from the reduction of 3.3 Gm. of phenylglyoxylohydroxamamide.

Reduction of Phenylglyoxylohydroxamyl Chloride.—Colorless crystals, melting at 209–210°, were obtained from the reduction of phenylglyoxylohydroxamyl chloride. This melting point agrees with that given in the literature for phenylethanolamine hydrochloride (4). The compound is water-soluble, giving a positive chloride ion test with aqueous silver nitrate test solution. The picrate softens at 154°, melting at 158° (decompd.). The melting point of the picrate of phenylethanolamine is reported at 154° in the literature. The yields obtained in three reductions were 86, 91, and 85%.

Anal.—Calcd. for $C_8H_{11}ON \cdot HCl$: N, 8.07%. Found: N, 8.37% and 8.47%.

Reduction of *p*-Xenylglyoxylohydroxamyl Chloride.—From the reduction of 5.2 Gm. (0.02 mole) of the chloride, in which 4 moles of hydrogen were absorbed in five hours, 4.1 Gm. (84%) of *p*-xenylethanolamine (*p*-phenylphenylethanolamine) hydrochloride was obtained, m. p. 191–192°. The picrate of this product melts at 114–115°.

[1] All reductions carried out at the Naval Research Laboratory, Washington, D. C.

Anal.—Calcd. for $C_{14}H_{15}ON \cdot HCl$: Cl, 14.06; N, 5.64%. Found: Cl, 13.44, 13.53%; N, 5.73% and 5.69%.

An aqueous solution of the hydrochloride was made alkaline and extracted with ether. The ethereal solution was dried over anhydrous $MgSO_4$ and evaporated to dryness in a desiccator under vacuum. A white solid, m. p. 126–129°, was obtained.

Reduction of *p*-Methylphenylglyoxylohydroxamyl Chloride. — *p* - Methylphenylglyoxylohydroxamyl chloride was reduced to the corresponding ethanolamine, four moles of hydrogen being absorbed in two and one-half hours. A white compound was obtained, which on recrystallization from hot absolute alcohol gave colorless platelets. From 3.9 Gm. (0.02 mole) of the chloride, 2 Gm. (54%) of colorless platelets, m. p. 189–190°, was obtained. The picrate melts at 155°.

Anal.—Calcd. for $C_9H_{13}ON \cdot HCl$: N, 7.49%. Found: N, 7.82% and 7.98%.

SUMMARY

1. The formation of arylglyoxylohydroxamamides from aliphatic and aromatic amines is described; the reaction has been applied to two aromatic amines, eight aliphatic amines, and to ammonia.

2. The hydrochlorides of some of these hydroxamamides have been prepared and utilized in characterizing the corresponding hydroxamamides.

3. Reduction studies of arylglyoxylohydroxamamides were undertaken. It has been found that the simple amide $C_6H_5 \cdot CO \cdot C(:NOH)NH_2$ takes up the calculated four moles of hydrogen to form the expected phenylethanolamine. The substituted amides take up but three moles, and indications are that in the product both nitrogen atoms are still present.

4. Arylglyoxylohydroxamyl chlorides have been successfully reduced to the corresponding amino alcohols by using a mixed palladium-platinum catalyst.

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(1) Levin, N., and Hartung, W. H., *J. Org. Chem.*, **7**, 408(1942).
(2) Hartung, W. H., Munch, J. C., Deckert, W. A., and Crossley, F., *J. Am. Chem. Soc.*, **52**, 3317(1930).
(3) Hartung, W. H., and Munch, J. C., *ibid.*, **51**, 2262 (1929).
(4) Mannich, C., and Thiele, E., *Arch. Pharm.*, **253**, 181 (1915).

The Preparation of Some Iodinated Phenyl- and Pyridylalkanoic Acids*,†

By S. ARCHER, J. O. HOPPE, T. R. LEWIS, and MARY N. HASKELL‡

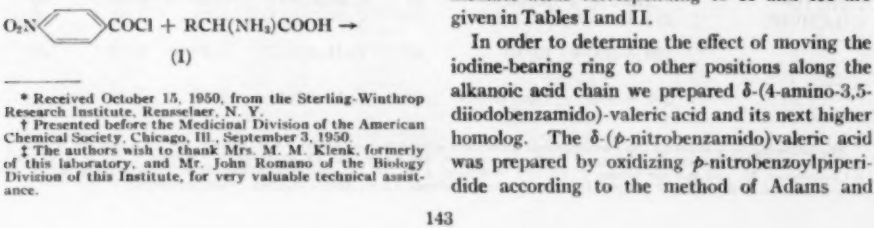
A series of aminodiiodophenylbenzamidoalkanoic acids was prepared by nitrobenzoylation of a group of amino acids, followed by reduction and iodination. Nine new aminodiiodophenylsulfonamidoalkanoic acids were prepared. 3,5-Diiodo-4-pyridone was allowed to react with a series of α-bromo acids and the corresponding diiodopyridone-N-alkanoic acids were studied for intravenous cholecystography. 3,5-Diiodo-4-pyridinethiol was condensed with a group of α-bromo acids. All the iodinated acids were evaluated as oral cholecystographic agents. An attempt has been made to point out the structural features that are necessary for a good gall-bladder contrast medium.

ALTHOUGH there is a moderate literature on the preparation and testing of cholecystographic agents, there have been few attempts made to define activity in terms of chemical structure (1, 2). In the present paper we describe three groups of potential gall-bladder contrast agents and try to point out the permissive and required features of compounds suitable for use in cholecystography.

Most of the substances which have been examined in recent years as cholecystographic agents have been diiodohydroxyphenylalkanoic acids (1, 2), diiodohydroxyphenylalkenoic acids (2), and aminodiiodophenylalkanoic acids (3–5). A diiodohydroxybenzylbenzoic acid has been studied by two groups of workers (6, 7). Jacobson and co-workers (8) prepared 3-iodo-7,12-diketocholanic acid and its glycine conjugate. Both failed to concentrate in the gall bladder. Iodoalphionic acid, which is used clinically under the trade name Priodax, is a diiodohydroxylphenylalkanoic acid, too (9).

We prepared a series of α-(3,5-diiodo-4-aminobenzamido)-alkanoic acids. The essential difference between this group of compounds and the one discussed previously (5) is the mode of linkage of the diiodoaminophenyl radical with the alkanoic acid residue. In the prior case (5) a methylene group served as the bridge, whereas here a carbamido unit joins the aromatic nucleus with the acid side chain.

The required acids were prepared according to Eq. 1. The amino acids (I) which were not obtained from commercial sources were synthesized from ethyl acetamidocyanoacetate and the requisite alkyl halide (10). Although Snyder, *et al.* (11), reported that they were unable to effect the condensation between secondary alkyl halides and ethyl acetamidomalonate, Albertson and Tullar (12) prepared valine from isopropyl bromide and ethyl acetamidocyanoacetate. We were able to condense both cyclohexyl and cyclopentyl bromide with ethyl acetamidocyanoacetate.



(I) (II) (III) (IV) (1)

The nitrobenzoylations of the amino acids were carried out in slightly alkaline solution. Occasionally we would observe the violet color which Karrer and Keller (13) attributed to the interaction of the alkali with an oxazolone. The reduction of the nitro group was performed catalytically with the aid of Raney nickel catalyst.

The iodination of the aminobenzamido acids (II) was accomplished with iodine monochloride in dilute acetic acid. The properties of the final products are listed in Table III. The intermediate acids corresponding to II and III are given in Tables I and II.

In order to determine the effect of moving the iodine-bearing ring to other positions along the alkanoic acid chain we prepared δ-(4-amino-3,5-diiodobenzamido)-valeric acid and its next higher homolog. The δ-(*p*-nitrobenzamido)valeric acid was prepared by oxidizing *p*-nitrobenzoylpiperidide according to the method of Adams and

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† Presented before the Medicinal Division of the American Chemical Society, Chicago, Ill., September 3, 1950.
‡ The authors wish to thank Mrs. M. M. Klenk, formerly of this laboratory, and Mr. John Romano of the Biology Division of this Institute, for very valuable technical assistance.

Leonard (14). The next higher homolog was obtained from ϵ-caprolactam by hydrolysis followed by nitrobenzoylation. Subsequent reduction and iodination steps were similar to those shown in Eq. 1.

We substituted a sulfonamido radical for the carbamido group in the above series. Klemme and Beals (15) prepared the first member of the series. We repeated their work and submitted the compound to cholecystographic testing. It

TABLE I.—PROPERTIES OF THE *p*-NITROBENZAMIDOALKANOIC ACIDS

R =	M. P., ° C., (Uncorr.)	Yield, %[a]	Formula	Analyses, % N— Calcd.	Found
$CH_3CH_2CH_2$	183–184	85	$C_{13}H_{14}N_2O_5$	10.52	10.51
$CH_3CH_2CH_2CH_2$	171–172	82	$C_{14}H_{16}N_2O_5$	10.00	10.47
$CH_3CH_2CH_2CH_2CH_2$	166–168	76	$C_{15}H_{18}N_2O_5$	4.77[b]	4.88
$(CH_3)_2CHCH_2CH_2$	197–198	56	$C_{15}H_{18}N_2O_5$	4.77[b]	4.73
	209–211	50	$C_{15}H_{13}N_2O_5$	4.57[b]	5.05
	205–207	93	$C_{14}H_{10}N_2O_5$	4.79[b]	5.03

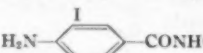
[a] All compounds were purified by crystallization from dilute ethanol.
[b] Nitro nitrogen determined by $TiCl_3$ reduction.

TABLE II.—PROPERTIES OF THE *p*-AMINOBENZAMIDOALKANOIC ACIDS

R =	M. P., ° C. (Uncorr.)	Yield, %	Solvent	Formula	Analyses, % N— Calcd.	Found
$CH_3CH_2CH_2$	163	80	Water	$C_{13}H_{18}N_2O_3$	11.86	11.58
$CH_3CH_2CH_2CH_2$	153–155	65	Dil. EtOH	$C_{14}H_{20}N_2O_3$	5.60[a]	5.45
$CH_3CH_2CH_2CH_2CH_2$	159–160	83	Dil. EtOH	$C_{15}H_{22}N_2O_3$	10.52	10.58
$(CH_3)_2CHCH_2CH_2$	187	64	Dil. EtOH	$C_{15}H_{22}N_2O_3$	10.82	10.47
	175–177	42	Dil. EtOH	$C_{13}H_{20}N_2O_3$	10.14	9.95
	223	70	Dil. EtOH	$C_{14}H_{18}N_2O_3$	5.30[a]	5.13

[a] Basic nitrogen by perchloric acid titration.

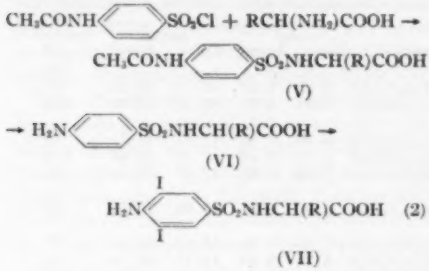
TABLE III.—PROPERTIES OF THE (4-AMINO-3,5-DIIODOBENZAMIDO)ALKANOIC ACIDS



R =	M. P., ° C. (Corr.)	Solvent	Yield, %	Formula	Analyses, % N— Calcd.	Found	Activity Priodax = 100
H[c]	224.8–225.4	Dil. EtOH	57	$C_9H_8I_2N_2O_3$	6.28	6.19	25
CH_3[b]	217	Dil. EtOH	53	$C_{10}H_{10}I_2N_2O_3$	6.09	6.00	25
$CH_3CH_2CH_2$	218–220	Dil. HOAc	82	$C_{12}H_{14}I_2N_2O_3$	5.74	5.59	25
$(CH_3)_2CH$[c]	237.5–238.2	Dil. EtOH	79	$C_{12}H_{14}I_2N_2O_3$	5.74	5.72	25
$CH_3CH_2CH_2CH_2$	184.5–185.2	Dil. HOAc	55	$C_{13}H_{16}I_2N_2O_3$	50.56[c]	50.30[c]	75
$(CH_3)_2CHCH_2$[d]	204.5–206	Dil. EtOH	63	$C_{13}H_{16}I_2N_2O_3$	5.58	5.32	40
$CH_3CH_2CH(CH_3)$[e]	223.4–223.6	EtOH	65	$C_{13}H_{16}I_2N_2O_3$	5.58	5.48	60
$CH_3CH_2CH_2CH_2CH_2$	159–166	Dil. EtOH	39	$C_{14}H_{18}I_2N_2O_3$	5.45	5.53	120
$(CH_3)_2CHCH_2CH_2$	163.4–164.6	EtOH	72	$C_{14}H_{18}I_2N_2O_3$	5.45	5.18	103
C_6H_5[f]	223–223.8	EtOH	66	$C_{15}H_{12}I_2N_2O_3$	5.38	5.14	0
$C_6H_5CH_2$[g]	216–216.5	HOAc	57	$C_{16}H_{14}I_2N_2O_3$	5.23	5.15	62
CH_3CHOH[h]	214.8–215.3	Dil. EtOH	50	$C_{11}H_{12}I_2N_2O_4$	5.79	5.53	0
	198.5–200.6	EtOH	50	$C_{15}H_{15}I_2N_2O_3$	5.30	5.15	20
	207.6–208	Dil. EtOH	56	$C_{16}H_{16}I_2N_2O_3$	5.45	5.26	

The following have reported the corresponding aminobenzamidoalkanoic acids: [c] Landsteiner, K., and van der Scheer, J., *J. Expil. Med.*, **55**, 781(1932); [b] Wright, W. B., Jr., *et al.*, *J. Am. Chem. Soc.*, **71**, 3014(1949); [c] Wright, J. B., Jr., *et al.*, *ibid.*; [d] Landsteiner, K., and van der Scheer, J., *loc. cit.*; [e] Wright, J. B., Jr., *loc. cit.*; [f] Ingersoll, A. W., and Adams, R., *J. Am. Chem. Soc.*, **44**, 2930(1922); [g] Wright, J. B., Jr., *loc. cit.*; [h] Maeda, *Chem. Abstr.*, **33**, 2945; [i] iodine analysis.

was a poor radiopaque. This is in accord with previous experience (1, 5). The higher homologs, which were more interesting pharmacologically, were prepared in the manner shown in Eq. 2.

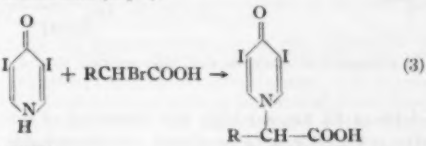


(V) (VI) (VII) (2)

Acetylaminobenzenesulfonyl chloride coupled readily with amino acids in basic solution to furnish compounds of formula V. Hydrolysis with dilute hydrochloric acid resulted in deacetylation and the free amino compounds, VI, were iodinated with iodine monochloride. The diiodo derivatives (VII) are listed in Table VI. Tables IV and V summarize the pertinent data on the series represented by V and VI, respectively.

In that series several members were poor radiopaques, but it was apparent from the films that the prime cause was that the drugs were not absorbed. To avoid this obstacle, we studied a series of intravenous cholecystographic media. Epstein noticed that the lower members of his series were eliminated by the kidney. The diethanolamine salt of 3,5-diiodo-4-pyridone-N-acetic acid is a nontoxic contrast agent which is used clinically for visualization of the kidney and ureters. It was hoped that the excretion characteristics of the higher homologs would be altered sufficiently so that at some point in the series gall-bladder visualization would result. Actually this proved to be the case (*vide infra*).

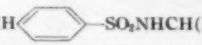
The series was prepared by condensing 3,5-diiodo-4-pyridone (16) with the appropriate α-bromo acid (Eq. 3).



(VIII) (IX) (3)

It has been established that N-alkylation occurs under the conditions of our experiments (16). The

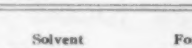
TABLE IV.—PROPERTIES OF THE *p*-ACETAMIDOBENZENESULFONAMIDOALKANOIC ACIDS



R = [a]	M. P., °C. (Uncorr.)	Formula	Analyses, % S Calcd.	Found
C_2H_5	190–191	$C_{12}H_{16}N_2O_5S$	10.68	10.88
$(CH_3)_2CH$	222–225	$C_{13}H_{18}N_2O_5S$	10.10	10.25
$CH_3CH_2CH_2CH_2$	190–191	$C_{14}H_{20}N_2O_5S$	9.76	9.82
$CH_3CH_2CH(CH_3)$	189–190	$C_{14}H_{20}N_2O_5S$	9.76	9.33
$(CH_3)_2CHCH_2$	211–212	$C_{14}H_{20}N_2O_5S$	8.53[b]	8.29
$CH_3CH_2CH_2CH_2CH_2$	168–170	$C_{15}H_{22}N_2O_5S$	9.36	9.46
$(CH_3)_2CHCH_2CH_2$	181–184	$C_{15}H_{22}N_2O_5S$	9.36	9.56
C_6H_5	224–226	$C_{16}H_{16}N_2O_5S$	9.20	9.23
$C_6H_5CH_2$	218–219	$C_{17}H_{18}N_2O_5S$	8.84	9.07
	238–240	$C_{13}H_{20}N_2O_5S$	9.41	9.55

[a] All compounds were purified by recrystallization from dilute ethanol.
[b] Nitrogen analysis.

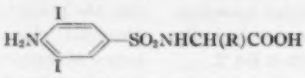
TABLE V.—PROPERTIES OF THE *p*-AMINOBENZENESULFONAMIDOALKANOIC ACIDS



R =	M. P., °C. (Uncorr.)	Solvent	Formula	Analyses, % S Calcd.	Found
C_2H_5	195–196	Water	$C_{10}H_{14}N_2O_4S$	12.41	12.51
$(CH_3)_2CH$	188–190	Water	$C_{11}H_{16}N_2O_4S$	11.77	11.73
$CH_3CH_2CH_2CH_2$	158–160	Water	$C_{12}H_{18}N_2O_4S$	4.89[a]	4.70
$CH_3CH_2CH(CH_3)$	172–173	Water	$C_{12}H_{18}N_2O_4S$	11.20	11.15
$(CH_3)_2CHCH_2$	192–194	Water	$C_{12}H_{18}N_2O_4S$	4.89[a]	4.62
$(CH_3)_2CHCH_2CH_2$	149–150	Dil. EtOH	$C_{13}H_{20}N_2O_4S$	10.67	10.87
C_6H_5	202–204	Dil. EtOH	$C_{14}H_{14}N_2O_4S$	10.47	10.61
$C_6H_5CH_2$	198.5–199	Dil. EtOH	$C_{15}H_{16}N_2O_4S$	8.75[b]	8.54

[a] Basic nitrogen by perchloric acid titration.
[b] Kjeldahl nitrogen.

TABLE VI.—PROPERTIES OF THE 4-AMINO-3,5-DIIODOBENZENESULFONAMIDOALKANOIC ACIDS



R =	M. P.° C. (Corr.)	Solvent	Formula	Analyses: Sulfur Calcd.	Sulfur Found	Iodine Calcd.	Iodine Found	Activity Priodax = 100
H[a]	...	...	...	...	...	...	...	32
C_2H_5	185.5–187	Dil. EtOH	$C_{10}H_{12}I_2N_2O_4S$	6.29	6.22	49.77	49.80	87
$(CH_3)_2CH$	179–181.1	EtOAc-ligroin	$C_{11}H_{14}I_2N_2O_4S$	6.11	6.27	48.46	48.00	108
$CH_3CH_2CH_2CH_3$	173–173.8	Dil. HOAc	$C_{12}H_{16}I_2N_2O_4S$	5.96	6.06	47.17	47.11	75
$CH_3CH_2CH(CH_3)$	169.5–170.5	Dil. HOAc	$C_{12}H_{16}I_2N_2O_4S$	5.96	6.10	47.17	46.60	110
$(CH_3)_2CHCH_2$	208.5–210.5	Dil. MeOH	$C_{12}H_{16}I_2N_2O_4S$	5.96	5.86	47.17	46.85	96
$CH_3CH_2CH_2CH_2CH_3$	176–178	Benzene-ligroin	$C_{13}H_{18}I_2N_2O_4S$	5.80	5.95	45.98	45.50	72
$(CH_3)_2CHCH_2CH_2$	194–195	Dil. EtOH	$C_{13}H_{18}I_2N_2O_4S$	5.80	5.89	45.98	46.00	71
C_6H_5	219.5–220	Dil. HOAc	$C_{16}H_{12}I_2N_2O_4S$	5.74	5.83	45.47	45.10	48
$C_6H_5CH_2$	197.4–199.4	HOAc	$C_{13}H_{14}I_2N_2O_4S$	5.60	5.70	44.33	44.85	102
	216–217.5	Dil. MeOH	$C_{13}H_{16}I_2N_2O_4S$	5.83	5.90	46.14	46.39	120

[a] Klemme, C. J., and Beals, E. L., reference 15.

yields of IX are not high, but almost all of unchanged VIII can be recovered. It is probable that dehydrobromination of the α-bromo acid is the side reaction responsible for the poor conversion. The compounds corresponding to IX are listed in Table VII.

A similar series was prepared from 3,5-diiodo-4-thiopyridone. Alkylation of X results in S- rather than N-alkylation (16). When heated with an α-bromo acid, compounds of formula XI were obtained (Eq. 4). After our work was completed, Klingsberg and Papa (17) reported the

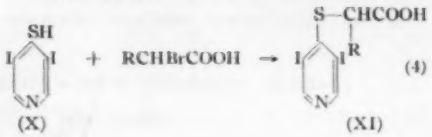


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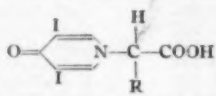
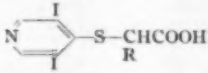
TABLE VII.—IODINATED PYRIDYLALKANOIC ACIDS



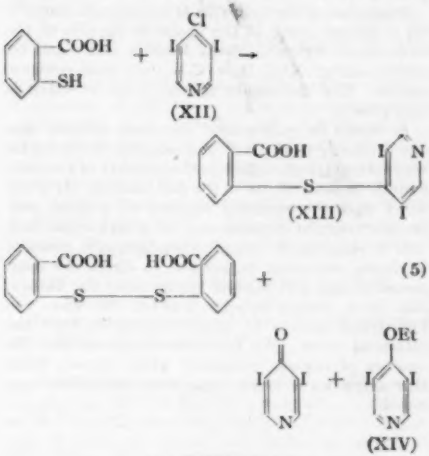
R =	M. P., ° C. (Corr.)	Solvent	Formula	Analyses: Nitrogen Calcd.	Nitrogen Found	Iodine Calcd.	Iodine Found	Activity, Priodax = 100
CH_3CH_2	168.4–169	EtOAc-ligroin	$C_9H_9I_2NO_2$	3.24	3.06	58.48	58.35	...
$CH_3(CH_2)_2$	194–197	Dil. EtOH	$C_{10}H_{11}I_2NO_2$	3.13	3.12	56.78	57.23	
$CH_3(CH_2)_3$	205–207	Dil. EtOH	$C_{11}H_{13}I_2NO_2$	3.04	3.05	55.05	54.97	...
$(CH_3)_2CHCH_2$	185.4–186.2	Dil. EtOH	$C_{11}H_{13}I_2NO_2$	3.04	2.92	55.05	54.60	...
$CH_3(CH_2)_4$	202.5–204.5	Dil. EtOH	$C_{12}H_{15}I_2NO_2$	2.95	2.92	53.43	54.00	...
$(CH_3)_2CHCH_2CH_2$	202–203.8	EtOH	$C_{12}H_{15}I_2NO_2$	2.95	2.76	53.43	53.90	...
$CH_3(CH_2)_5$	176.2–177	EtOH	$C_{13}H_{17}I_2NO_2$	2.86	2.88	51.90	51.40	...
$CH_3(CH_2)_6$	132–133	Ether-pentane	$C_{14}H_{19}I_2NO_2$	2.78	2.69	50.45	50.34	...
$CH_3(CH_2)_7$	124.5–126.5	Dil. EtOH	$C_{15}H_{21}I_2NO_2$	2.71	2.66	49.08	49.08	...



				Sulphur Calcd.	Sulphur Found			
$CH_3CH_2CH_2$	152.6–153.4	Toluene-ligroin	$C_{10}H_{11}I_2NO_2S$	6.92	7.20	54.93	55.20	112
$CH_3CH_2CH_2CH_2$	153.1–155.9	Benzene-ligroin	$C_{11}H_{13}I_2NO_2S$	6.90	6.70	53.10	53.20	120
$(CH_3)_2CHCH_2CH_2$	134.5–136.2	Benzene-ligroin	$C_{12}H_{15}I_2NO_2S$	6.53	6.67	51.68	51.25	96

preparation of the first member of the series. They noted that XI (R = H) was unstable in solution and could not be crystallized in quantity. We did not observe this instability with the higher members of the series. The compounds were readily purified by crystallization and good cholecystograms were obtained on oral administration. The compounds are listed in Table VII.

When thiosalicylic acid was treated with 3,5-diiodo-4-chloropyridine in sodium ethylate solution, a complex mixture resulted. The desired 2-carboxyphenyl 3,5-diiodo-4-pyridyl sulfide (XIII) was secured in low yield, together with dithiosalicylic acid, 3,5-diiodo-4-pyridone and 3,5-diiodo-4-ethoxypyridine (XIV) (Eq. 5). The

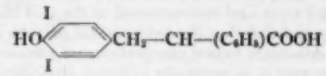


acid XIII was isolated and purified as the piperidine salt. The piperidine salt of the dithiosalicylic acid was compared with an authentic sample and found to be identical. The structure of the ether, XIV, was proved by synthesis. The chloropyridine, XII, was heated in sodium ethylate solution to furnish an authentic specimen of XIV.

The dithiosalicylic acid and the diiodopyridone may have arisen from the decomposition of XIII in a way similar to that described by Klingsberg and Papa (17). In the present case the disulfide linkage is found in the acid moiety rather than in the pyridine portion of the molecule.

PHARMACOLOGY

The compounds discussed above and in previous papers (3–5) were screened orally in cats. These animals were selected because reproducible results could be obtained, their size tended to conserve the amount of drug required, and a relatively large number of compounds could be screened in a short period of time. A standard series of films was obtained first with Priodax, XV (below). Shadows were rated as



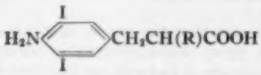
(XV)

absent, poor, fair, good, and excellent. Numerical values were assigned to the cholecystograms to facilitate comparing activities. Ratings were then made in terms of the standard, XV. In preliminary tests usually the 100 mg./Kg. dose was used for comparison.

In the case of the α-(3,5-diiodo-4-pyridone)-N-alkanoic acids, the substances were administered as their diethanolamine salts. The toxicities were found to vary directly with chain length so that only those compounds containing seven or less carbon atoms in the alkanoic acid portion of the molecule were used for cholecystography.

In the benzamido series very poor delineation of the gall bladder was obtained when the side chain (R) contained less than four carbon atoms. α-(4-Amino-3,5-diiodobenzamido)phenylacetic acid and its hexahydro derivative were almost devoid of cholecystographic activity due to lack of absorption. The α-(4-amino-3,5-diiodobenzamido)-heptanoic acids showed maximal activity in the series. Here the *n*-heptanoic acid was somewhat better than the iso acid. The isocaproic acid was better than the corresponding straight chain isomer, too. The effect of moving the diiodoaminobenzamido group from the α-position to the end of the chain was most striking. Both ε-(4-amino-3,5-diiodobenzamido)-caproic acid and the next lower homolog failed to concentrate in the gall bladder.

A comparison with the aminodiiodophenylalkanoic acid series is of interest (5). In the group represented by formula XVI, maximum activity was reached



(XVI)

when R = C_4H_8 rather than C_5H_{11}. The amyl homolog in this series was a decidedly weaker radiopaque. When R = C_6H_8 shadows of great intensity equal to Priodax were obtained. In both series when R = cyclohexyl, poor concentration was observed.

The sulfonamido series as a whole was more active than the carbamido series. Peak activity was reached when the side chains contained three to five carbon atoms (Table VI). The radiopaque derived from phenylglycine was a fair radiopaque and the derivative of phenylalanine rivaled Priodax in activity. In this group the straight chain isomers appeared to be somewhat better than the branched chain compounds.

In contrast to the diiodopyridone-alkanoic acids, which were not absorbed when administered orally, the group containing the diiodopyridylthio radical did pass through the intestinal tract and concentrated in the gall bladder. Compound XI (R = C_4H_9) was one of the most active encountered in our studies.

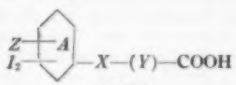
DISCUSSION

In order that a cholecystographic contrast medium be orally effective, it must be absorbed from the intestinal tract and concentrated in the gall bladder in such condition that the iodine is still present in the drug. Aromatic iodine compounds are customarily used because it is generally believed that these are less affected by metabolic processes than aliphatic iodine derivatives.

The lack of absorption of a drug does not reflect its ability to concentrate in the gall bladder. Thus, the diiodopyridonealkanoic acids passed through the intestinal tract unabsorbed. When the less toxic members were injected intravenously, concentration either in the gall bladder or by the kidney occurred, depending upon the side chain length. The butyric acid (IX, R = C_3H_5) appeared in the urinary bladder. The valeric acid (IX, R = C_3H_7) visualized both the gall bladder and urinary bladder. The caproic and heptanoic acids appeared only in the gall bladder. Very good contrast was obtained with the latter.

These results parallel those of Epstein (1). The lower members of his series were eliminated by the kidneys, the middle members were good gall-bladder contrast agents, and the higher ones were unabsorbed.

Most of the compounds studied by us and others can be formulated as members of a general class represented by XVII.



(XVII)

where

Z = NH_2 or OH
X = CH_2, S, CONH, SO_2NH, CH=CH
Y = lipophilic group
A = aromatic ring

e have seen that the nature of Z has relatively little effect upon cholecystographic potency (2–5). Its prime purpose is to facilitate the introduction of the iodine atoms into the aromatic ring. When this can be done by some other means as in the case of the diiodopyridylthioalkanoic acids (XI), good cholecystographic agents may still be obtained. This group of compounds is particularly useful since it demonstrates that both the hydroxyl and amino groups are not necessary for good visualization, and, further, that a phenyl group may also be replaced by some other iodine-bearing nucleus. Thus the function of the aromatic ring is to act as the halogen carrier.

The nature of the lipophilic group, Y, is of extreme importance. It is the variation in this portion of the molecule which exerts the most profound effect upon the potency of the contrast medium. When Y is too large, as in the case of Epstein's higher members, no absorption occurs. When it is too small, either the drug is not absorbed or it is eliminated by the kidneys. Jacobson's iodocholanic acid (8) does not fit into the general formula XVII because of the absence of an aromatic ring but the group corresponding to Y (the steroid nucleus plus side chain) is much too large for good activity.

Variation in the nature of the bridge, X, causes quantitative rather than any deep-seated qualitative effects on cholecystographic activity. When X is sulfonamido, maximum activity is reached when the side chain is butyl (XII, R = C_4H_9). In the carbamido series the peak is reached at the five carbon state (IV, R = C_5H_{11}).

In general it may be stated that, in compounds containing diiodophenyl or diiodopyridyl rings, maximum activity may be expected when the lipophilic group, Y, in formula XVII contains between three and eight carbon atoms, depending upon the nature of Z and X. Recently, Epstein, Cohen, and Natelson (18) reported that 2-(3,5-diiodo-4-hydroxybenzyl)-3,6-dimethylbenzoic acid was a good cholecystographic medium. In this compound the lipophilic portion Y contains eight carbon atoms, which is just at the upper limit we set in our loose generalization.

Prediction of the behavior of compounds containing a phenyl group in the lipophilic portion of the molecule is difficult. Both Priodax (XV) and its amino analog (XVI, R = C_6H_5) are good contrast agents. The carbamido analog (V, R = C_6H_5) is very poor.

It should be emphasized that poor activity was due primarily to the lack of absorption from the intestinal tract, rather than to the inability of the compounds to concentrate in the gall bladder. It is our belief that the essential features of a good gall-bladder contrast medium are: (*a*) a high iodine content to visualize the organ; (*b*) a lipophilic group of moderate size must be present to direct the compound to the gall bladder rather than the kidney, and (*c*) a proper balance between the lipo- and hydrophilic groups to insure absorption from the intestinal tract. We have demonstrated that the number of organic molecules which possess these characteristics is much larger than heretofore supposed.

EXPERIMENTAL

δ-(*p*-Nitrobenzamido)valeric Acid.—A solution of 38.0 Gm. of *p*-nitrobenzoylpiperidide in 2.5 L. of water was heated to boiling and then treated with a suspension of 57 Gm. of potassium permanganate in 500 ml. of water over a one-hour period. The alkaline solution was filtered hot and acidified to Congo red. The crystals that separated were filtered and dissolved in dilute sodium carbonate solution. After filtration, the solution was carefully acidified, the solid collected on a filter and washed with water.

The crops obtained from the oxidation of 100 Gm. of the piperidide were combined and recrystallized from boiling water. Yield: 51.3 Gm., or 45% of the theoretical. M. p. 159° (uncorr.).

Anal.—Calcd. for $C_{12}H_{14}N_2O_5$: N, 10.52. Found: N, 10.68.

δ-(*p*-Aminobenzamido)valeric Acid.—The following general procedure was used to prepare all the aminobenzamidoalkanoic acids. A solution of 51.3 Gm. of the above nitro acid in 300 ml. of methanol was shaken with hydrogen at room temperature in the presence of Raney nickel catalyst. After the theoretical amount of hydrogen was absorbed, the solution was filtered. The filtrate was concentrated to dryness and the residue was recrystallized from

hot water, m. p. 135–137° (uncorr.). Wt. 35.0 Gm., or 74% of the theoretical yield.

Anal.—Calcd. for $C_{12}H_{16}N_2O_3$: N, 11.43. Found: N, 11.40.

δ-(4-Amino-3,5-diiodobenzamido)valeric Acid.—A solution of 35 Gm. of the above acid in 100 ml. of glacial acetic acid and 50 ml. of water was heated to 70° and treated with 15 ml. of iodine monochloride in 50 ml. of glacial acetic acid over a period of one hour. Then 100 ml. of water was added slowly. The mixture was cooled and filtered. The product was crystallized from dilute acetic acid twice. Finally, the acid was dissolved in dilute sodium bicarbonate and the solution was acidified with sulfur dioxide. The almost colorless crystals were filtered, washed with water, and dried. M. p. 196.8 to 197.4° (corr.). Yield: 42 Gm. (50% of the theory).

Anal.—Calcd. for $C_{12}H_{14}I_2N_2O_3$: C, 29.63; H, 2.90; N, 5.76. Found: C, 29.48; H, 2.73; N, 5.69.

ε-(4-Amino-3,5-diiodobenzamido)caproic Acid.—A solution of 116 Gm. of ε-(4-aminobenzamido)-caproic acid, m. p. 128–130° (19), was dissolved in a mixture of 250 ml. of acetic acid and 50 ml. of water, and iodinated as above with 45 ml. of iodine monochloride in 200 ml. of acetic acid. The product was recrystallized twice from dilute acetic acid and weighed 134 Gm. (67%). After one more recrystallization, the pure acid melted at 165.2 to 166.8° (corr).

Anal.—Calcd. for $C_{13}H_{16}I_2N_2O_3$: I, 50.40; N, 5.57. Found: I, 50.74; N, 5.39.

The compounds listed in Table III were iodinated according to the same procedure.

α-(*p*-Nitrobenzamido)valeric Acid.—The following is a typical nitrobenzoylation procedure. A solution of 39.5 Gm. of norvaline in 250 ml. of water containing 10 Gm. of sodium hydroxide was cooled to 20°. Then 47 Gm. of *p*-nitrobenzoyl chloride was added portionwise to the stirred solution. The mixture was kept at *p*H 8–10 by the occasional addition of 2 *N* sodium hydroxide. After one-half hour the mixture was filtered. The filtrate was acidified, and the product collected and recrystallized from dilute ethanol. It melted at 183–184° (uncorr.) and weighed 57 Gm. (85%).

α-Aminocyclopentylacetic Acid.—A solution of 34.5 Gm. of sodium and 255 Gm. of ethylacetamidocyanoacetate in 750 ml. of absolute alcohol was heated to boiling. To the vigorously stirred solution, 225 Gm. of cyclopentyl bromide was added dropwise over a half-hour period. The mixture was refluxed for seventeen hours and poured into ice water. The solid was filtered and pressed as dry as possible.

It was suspended in 2,500 ml. of 10% sodium hydroxide and refluxed overnight. The solution was made faintly acidic and cooled. The amino acid was dissolved in dilute hydrochloric acid, boiled with Darco, and filtered. The hot solution was heated with a slight excess of pyridine, cooled, and filtered. After washing with alcohol and drying, there was obtained 66.5 Gm. of the desired product (50% yield). M. p. >220°.

Anal.—Calcd. for $C_7H_{13}NO_2$: N, 9.79. Found: N, 9.79.

α-Aminocyclohexylacetic Acid.—This acid was obtained in 30% over-all yield by the above procedure. The crude amino acid was used in the nitrobenzoylation step.

α-(3,5-Diiodo-4-oxopyridyl)caprylic Acid.—The following method is typical for the preparation of the oxo-pyridyl compounds listed in Table VII. A solution of 35.0 Gm. of 3,5-diiodo-4-oxypyridine in 300 ml. of water containing 5.0 Gm. of sodium hydroxide was heated to 90°. To the resulting clear solution there was added a solution of 8.0 Gm. of sodium hydroxide in 300 ml. of water, in which there was dissolved 25.0 Gm. of α-bromocaprylic acid. The mixture was heated for two hours on the steam bath. At the end of this time it was cooled, and carbon dioxide was bubbled in until the solution was acid to phenolphthalein. The solid, which was unreacted diiodopyridone, was filtered off, washed with water, and dried. It weighed 21.8 Gm.

The filtrate was made strongly acid to precipitate a gum which solidified on standing. It was collected and dried. It then weighed 18.0 Gm. After recrystallization from dilute ethanol with the aid of Darco, the white crystals weighed 15.0 Gm. and melted at 176.2 to 177° (corr.). The analytical data and other properties of the series are listed in Table VII.

α-(3,5-Diiodopyridyl 4-thio)valeric Acid.—A solution of 18.0 Gm. of 3,5-diiodo-4-pyridenethiol and 9.0 Gm. of α-bromovaleric acid in 100 ml. of 1 *N* NaOH was stirred and heated on the steam bath for two hours. The solution was cooled and sulfur dioxide was passed in. The yellow crystals were filtered off and recrystallized from dilute ethanol. The needles were filtered. Wt. 17.0 Gm. (74% of the theoretical). The product was then recrystallized from toluene-ligroin.

α-Carboxyphenyl-4-(3,5-diiodopyridyl)sulfide.—A solution of 2.3 Gm. of sodium in 500 ml. of absolute alcohol was heated to boiling and then 7.6 Gm. of thiosalicylic acid and 18.0 Gm. of 3,5-diiodo-4-chloropyridine was added. The mixture was stirred under reflux overnight. The alcohol was removed *in vacuo* and the residue was treated with hot dilute sodium hydroxide. The insoluble solid (A) was filtered off. On cooling, the solution deposited a new product (B). This was removed and the filtrate was acidified. The solid that separated was collected and boiled with alcohol. An insoluble product (C) was filtered off. Dilution of the mother liquor with water afforded (D)

The solid A was recrystallized from alcohol-water. Wt. 3.7 Gm. On further recrystallization it melted at 56.1 to 56.9° (corr.). It was 3,5-diiodo-4-ethoxypyridine.

Anal.—Calcd. for $C_7H_7I_2NO$: I, 67.51; N, 3.72. Found: I, 67.50; N, 3.75.

Product B was dissolved in ethanol and acidified to give a yellow solid. Wt. 4.7 Gm. It was dissolved in 250 ml. of absolute alcohol and treated with a slight excess of piperidine. On cooling there was obtained 3.6 Gm. of the piperidinium salt of 2-carboxyphenyl-4-(3,5-diiodopyridyl)sulfide. M. p. 229–231°.

Anal.—Calcd. for $C_{12}H_7I_2NO_2S \cdot C_5H_{11}N$: C, 35.90; H, 3.17; I, 44.67; N, 4.93. Found: C, 35.60; H, 3.14; I, 43.80; N, 4.97.

Product C was taken up in dilute sodium hydroxide and precipitated by passing carbon dioxide into the solution. It was filtered and dried. Wt. 2.5 Gm. It did not melt below 250°. It was probably 3,5-diiodo-4-pyridone.

Product D which weighed 1.5 Gm. did not melt below 280°. It formed a piperidine salt which melted at 205–209°. It did not depress the melting point of an authentic sample of the piperidine salt of dithiosalicyclic acid, m. p. 213–215°.

3,5-Diiodo-4-ethoxypyridine.—A solution of 0.25 Gm. of sodium and 4.0 Gm. of 3,5-diiodo-4-chloropyridine in 50 ml. absolute alcohol was refluxed for three hours. The mixture was poured on ice and the oil that resulted was treated with 2 *N* sodium hydroxide. The insoluble material was recrystallized from ethanol. Wt. 1.3 Gm. After one more recrystallization it melted at 56.2 to 57.4°. It did not depress the melting point of product A above.

α-(*p*-Acetamidobenzenesulfonamido)-β-phenylpropionic Acid.—The following is a typical procedure used in the condensation of the amino acids with *p*-acetaminobenzenesulfonyl chloride.

To a well-stirred solution of 50 Gm. of phenylalanine in 300 ml. of 2 *N* sodium hydroxide there was added 70 Gm. of *p*-acetylaminobenzenesulfonyl chloride in small portions. After several hours a salt started to separate (in the case of the more soluble amino acids the solution remained clear). An equal volume of water was added to dissolve the salt and the mixture was filtered to remove unreacted sulfonyl chloride. The filtrate was acidified with hydrochloric acid and the solid that separated was collected. After recrystallization from dilute alcohol there was obtained 70 Gm. of product. The other derivatives are listed in Table IV.

α-(*p*-Aminobenzenesulfonamido)-β-phenylpropionic Acid.—A suspension of 70 Gm. of the above acid, 35 ml. of concentrated hydrochloric acid, and 200 ml. of water was heated under reflux for about thirty minutes or until all the solid had dissolved. The solution was made almost neutral with dilute potassium hydroxide and cooled. The solid was filtered and recrystallized from dilute ethanol. Wt. 54 Gm. This procedure was used for hydrolyzing all the acetamido compounds. The derivatives are listed in Table V.

α-(p-Amino-3,5-diiodobenzenesulfonamido)-β-phenylpropionic Acid.—The following is the iodination procedure used for the acids listed in Table VI.

Fifty-four grams of α-(4-aminobenzenesulfonamido)-β-phenylpropionic acid was suspended in 340 ml. of acetic acid and heated to 80°. Then 18.2 ml. of iodine monochloride was added in one portion followed by 115 ml. of water. The purple mixture was stirred and heated for one hour and then allowed to stand overnight. It was filtered and recrystallized from dilute acetic acid.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

p-Hydroxyhippuric acid
Uramildiacetic acid
Ferritin
Toxisterol
L-Xylulose
Hexokinase
3-Hydroxycholinic acid
Thymidylic acid
Isolysergic acid
Trimethyl-(*p*-aminophenyl)ammonium chloride
Indigo brown
Vulbocapnine
Primula acid
Phenamidine
Copper pyridine thiocyanate
Diadzin
Quercitagetin
Myricetin

Micro-Kjeldahl Method for Biologicals*

By MORRIS B. JACOBS

The micro-Kjeldahl methods described in the literature are ambiguous with particular reference to the amount of potassium sulfate to be used. If precision and accuracy are to be obtained with micro variation of the Kjeldahl method in which potassium sulfate is employed, it is necessary to use a minimum of 0.7 to 0.8 Gm. of potassium sulfate per ml. of concentrated sulfuric acid. Results showing the inadequacy of lesser amounts are detailed. By use of a single reagent consisting of a solution of potassium sulfate, copper sulfate, and sulfuric acid in water, the manipulations of the micro-Kjeldahl method can be reduced and thus simplified. The results achieved with this reagent in the micro-Kjeldahl method indicate that it is equivalent in precision and accuracy to other variations of the method.

THE CONDITIONS required for complete recovery of nitrogen by modifications of the Kjeldahl method have been reviewed in considerable detail, particularly for proteinaceous material, by Kirk (4, 5). There is no need to repeat this information here. It is necessary to point out, however, that there is no general agreement on these conditions, either for the macro- or micro-Kjeldahl variations.

It is the purpose of this paper to present data concerning the use of potassium sulfate as one of the reagents in the Parnas-Wagner (7) modification of the Pregl (8, 9) modification of the Kjeldahl method. The Pregl-Parnas-Wagner modification is one of the most commonly used for the determination of nitrogen in biologicals, and the details usually followed are those given in the books of Pregl (8, 9) or of Niederl and Niederl (6). The descriptions in these texts, particularly with respect to the amount of potassium sulfate to be used, is not at all clear. Thus, in the former, one reads the statement, " . . . a knife point of selenium or potassium sulfate and copper sulfate mixture is added . . . " (the mixture being 1 of potassium sulfate to 3 of copper sulfate!) and in the latter the directions are even more ambiguous. Kabat and Mayer (3) are somewhat more definite, calling for approximately 1 Gm. of potassium sulfate in one instance (page 284, first edition) and for approximately 0.25 Gm. in another modification (page 287, first edition).

It has been pointed out by Willits, Coe, and Ogg (12) that there is a great danger of the loss of nitrogen when selenium is present. They do not recommend the use of selenium as an additional catalyst.

It has been the experience of the author that in order to obtain precision by the Pregl-Parnas-Wagner method it is essential to add a proper amount of potassium sulfate, and preferably to add the same amount of it for each determination. This can be achieved by use of a single reagent. It is a relatively common practice to saturate the sulfuric acid used with copper sulfate and then add a known volume of this sulfuric acid-copper sulfate reagent. Other analysts have mixed copper sulfate and potassium sulfate as powders, and have added various amounts of this mixture. The use of a single reagent seems to have been neglected in the literature.

It has been our experience that an aqueous solution containing the proper amounts of sulfuric acid, potassium sulfate, and copper sulfate can be prepared which enables the analyst to obtain consistent results in the analysis of biologicals.

The term biologicals, as used in this paper, is meant to apply to such products as toxins, toxoids, antitoxins, and vaccines which are used for therapeutic or experimental purposes.

PREPARATION OF REAGENT

Dissolve 6 Gm. of recrystallized copper sulfate, $CuSO_4 \cdot 5H_2O$ and 105 Gm. of potassium sulfate, K_2SO_4, in 600 ml. of water by bringing the mixture to boiling. Cool approximately to room temperature and cautiously add 150 ml. of concentrated sulfuric acid. Cool again to room temperature, transfer to a graduated cylinder, and make up to a total volume of 900 ml.

The results obtained with this reagent will be detailed and discussed below.

METHOD

In this method, which is a modification of the Pregl-Parnas-Wagner method, protein and other forms of organic nitrogen are converted to ammonia and fixed as ammonium sulfate by digestion with sulfuric acid. Potassium sulfate is used to raise the temperature of the digestion mixture, and copper sulfate is used as the catalyst. The ammonia formed is liberated by the addition of sodium hydroxide solution, is steam-distilled, trapped in standard hydrochloric acid solution, and the excess hydrochloric acid is estimated titrimetrically with

* Received May 18, 1950, from the Bureau of Laboratories, Department of Health, City of New York.

standard sodium hydroxide solution, or the ammonia is trapped in boric acid solution and the amount is determined by direct titration with standard hydrochloric acid.

The apparatus is that of Parnas and Wagner (7) or the modification of this apparatus described by Shepard and Jacobs (10).

Procedure.—Dilute an aliquot portion of the material being analyzed, if necessary, to a known volume in a volumetric flask, so that the amount of nitrogen will be of the order of 0.5 mg./ml. Transfer 1 ml. of this solution to a micro-Kjeldahl digestion flask. With the aid of a burette or similar device, add 1 ml. of concentrated sulfuric acid, and 1 ml. of concentrated sulfuric acid, and 1 ml. of a 4% copper sulfate solution, prepared by dissolving 4 Gm. of $CuSO_4 \cdot 5H_2O$ in water and diluting to 100 ml. Add x gram of potassium sulfate (as noted below), mix, and digest on a digestion oven. Raise the heat slowly, boil vigorously, and after the material has been digested, as evidenced by a clear, straw yellow or light green color, reduce and cut off the heat. This process generally takes about twenty minutes. If the mixture does not clear in this time, reduce the heat, carefully add two to three drops of 30% hydrogen peroxide solution, and then continue heating for five to ten minutes. Allow to cool, add 4 ml. of water, and stir to dissolve the salts.

Add 7.0 ml. of 0.01 *N* hydrochloric acid, accurately measured, to a 25-ml. flask and add a trace of methyl red indicator solution. Allow the water in the steam generator to boil gently, remove the clamp from the line leading from the steam generator to the vapor vessel, and open the pinch clamp or stopcock at the bottom of the steam vessel so that the steam can escape. Transfer the digest from the micro-Kjeldahl digestion flask to the distillation tube through the small funnel. Wash out the micro-Kjeldahl digestion flask with two 2-ml. portions of distilled water and add these washings to the distillation tube. Place the receiving flask under the condenser so that the tip of the silver tube condenser is below the standard acid. With the aid of a pipette, add 7 ml. of 30% sodium hydroxide solution to the mixture in the distillation tube through the small funnel.

Close the stopcocks or pinch clamps of the small funnel, the vapor vessel, and the steam escape, if one is used, thus compelling the steam to pass through the distillation tube. Distill for exactly three minutes. Lower the receiving flask so that the tip of the condenser is about 1 cm. above the surface of the distillate. Continue the distillation for another minute. Rinse the tip of the condenser tube with a few drops of water. Add another trace of methyl red indicator solution, if this is necessary. Titrate with standard 0.01 *N* sodium hydroxide solution. One milliliter of standard hydrochloric acid (0.01 *N*) is equivalent to 0.14 mg. of nitrogen. Run a blank and subtract the blank from the volume of standard hydrochloric acid used.

Instead of using a known volume of standard hydrochloric acid, trap the ammonia being distilled in 10 ml. of cold 2% boric acid solution (2, 11). Proceed with the method as detailed, except, titrate the boric acid solution directly with 0.01 *N* hydrochloric acid. Run a blank and subtract this from the volume of standard hydrochloric acid used.

Procedure with Single Reagent.—The method as detailed above, and the washing procedure and apparatus described in the paper of Shepard and Jacobs (10) was followed and used, with the exception that 6, 7, 8, and 9 ml. of the prepared digestion reagent were used in place of the 1 ml. of sulfuric acid and the weighed amounts of potassium sulfate and copper sulfate.

Because of the increase in volume of the material being digested, attributable to the volume of reagent added, it is necessary to heat the mixture cautiously to prevent excessive frothing while the water is being driven off. Small sections of melting-point tubes or glass beads may be used to avoid bumping and decrease frothing, but care should be exercised not to permit them to get into the distillation tube.

EXPERIMENTAL RESULTS

The method was used to evaluate the "nitrogen" of crude bacterial filtrates of diphtheria toxoid and tetanus toxoid prepared with customary and semi-synthetic media. For the particular results detailed, two master batches of diphtheria toxoid were used. In addition, recrystallized glycine was used as a standard reference substance. The 0.01 *N* hydrochloric acid was standardized against standard 0.01 *N* sodium hydroxide solution, which was standardized against potassium acid phthalate, as the primary standard.

The results obtained by the method in which weighed portions of crystalline potassium sulfate were used are detailed in Table I and Fig. 1.

As representative of the results obtained with the single reagent, and for comparison with the results obtained with the variation of the method in which crystalline potassium sulfate was used, the data obtained with diphtheria toxoid sample MS-16 are detailed in Table II.

It is to be noted that the grand average shown by the variation, in which the potassium sulfate was weighed before addition to the digestion mixture, was 2.26 mg. nitrogen per ml., and that given by the method using a single reagent was 2.25 mg. nitrogen per ml.

TABLE I.—EFFECT OF POTASSIUM SULFATE CONCENTRATION ON APPARENT NITROGEN CONTENT[a]

K_2SO_4, Gm.	1	2	3	4	5	Av.
0.0	1.90	1.99	1.97	1.98	...	1.96
0.1	1.85	1.85	2.10	2.13	...	1.98
0.2	2.04	2.05	2.12	1.99	...	2.05
0.3	1.99	2.11	2.12	...	...	2.07
0.4	1.97	1.93	2.11	2.20	...	2.08
0.5	1.98	2.11	2.15	...	...	2.10
0.6	2.10	2.11	2.20	...	...	2.14
0.7	2.28	2.25	2.24	2.22	...	2.25
0.8	2.26	2.23	...	...	...	2.25
0.9	2.15	2.27	2.28	...	...	2.23
1.0	2.17	2.29	2.29	2.28	...	2.26
1.1	2.22	2.22	2.29	...	...	2.24
1.2	2.25	2.32	2.34	2.17	2.32	2.26
1.3	2.34	2.27	2.33	2.33	...	2.32
1.4	2.25	2.28	2.25	2.30	...	2.27
1.5	2.20	2.27	...	...	...	2.24
	Av. 0.7 through 1.5 Gm. K_2SO_4					2.26

[a] Milligrams per ml. (sample MS-16 diphtheria toxoid).

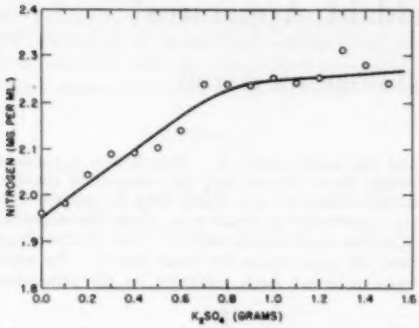

Fig. 1.—Effect of potassium sulfate concentration on apparent nitrogen content.

DISCUSSION

These results indicate that if less than 0.7 Gm. of potassium sulfate is used with the method as detailed full recovery of nitrogen is not obtained. The amount of apparent nitrogen rises asymptotically with increase of the amount of potassium sulfate used, and then remains substantially the same in the range of 0.8 to 1.3 Gm. There is a suggestion that the amount of apparent nitrogen will diminish if higher amounts of potassium sulfate are used. This has been discussed by Kirk (5).

When lower amounts of potassium sulfate were used, there was invariably difficulty in obtaining a clear digest within twenty minutes. Sometimes no clear digest was obtained even on continued heating. Actually no hydrogen peroxide was used in these experiments since it was desired not to confuse the issue concerning the role of potassium sulfate. The darker digests were distilled in the usual manner.

The experience of the author with thousands of determinations performed on food samples and on biologicals (1) both on a macro and micro scale indicates that (*a*) the macro Kjeldahl-Gunning-Arnold method as modified by Jacobs (2) appeared to be the simplest and an adequate variation for macro samples, and (*b*) this variation

TABLE II.—NITROGEN CONTENT OF DIPHTHERIA TOXOID[a]

Digestion Reagent, Ml.	Equivalent K_2SO_4, Gm.	1	2	3	Av.
6	0.7	2.26	2.17		
7	0.82	2.31	2.27	2.27	
8	0.93	2.27	2.29		
9	1.17	2.17	2.20		
					2.25

[a] Milligrams per ml.

adapted for the Pregl-Parnas-Wagner modification is adequate for the micro scale. It is essential, if accuracy and precision are to be obtained, to use the proper amount of potassium sulfate in the variations of the Kjeldahl method discussed.

The single reagent has been in use for over a year and one-half. Its efficacy was checked in two ways: first, by using it in the digestion of a material of known purity and composition, namely, recrystallized glycine, and second, by using it in the digestion of materials analyzed by other variations of the micro-Kjeldahl method. The results indicate that this method is equal to the others in accuracy and precision. While there is a slight increase in the time of digestion, since it is necessary to drive off the additional water of the reagent with care, it is still to be preferred because of the greater ease of manipulation.

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Modified Micro-Kjeldahl Apparatus*

By DAVID L. SHEPARD and MORRIS B. JACOBS

By placing a steam escape in the stopper of the steam generator of the Parnas-Wagner micro-Kjeldahl apparatus, a marked reduction in the time required for preparing the apparatus for the succeeding determination is achieved. The distillation and washing procedure using this modified apparatus is illustrated and detailed.

ALTHOUGH there have been numerous modifications of the micro-Kjeldahl nitrogen distillation apparatus used for the Pregl method, namely, those of Parnas and Wagner (13), Kemmerer and Hallett (8), Cavett (3), Andersen and Jensen (1), Groak (7), Nash (12), Kirk (9), Fife (6), Zakrzewski and Fuchs (16), Das Gupta (4), Redemann (14), Meyer (11), Brant and Sievers (2), and Markham (10), and several offered by firms dealing in specialized chemical glassware (5, 15), nevertheless the apparatus suggested by Wagner and Parnas is one of the most commonly employed. This apparatus has one marked disadvantage which has led in some measure to the development of the aforementioned different devices.

EXPERIMENTAL

In order to create the vacuum necessary so that the mixture being distilled, i. e., the spent material and also the wash water, can be drawn back by suction, it is necessary to remove the flame from under the steam generator (*G* in Fig. 1). Then the flame must be replaced to reheat the water in the steam generator to boiling in order to wash the apparatus or to make the next determination. Such a process results in considerable loss of time.

By a very simple modification of the original apparatus (shown in Fig. 1) which can be made by any technician, a constant boiling device is created, by means of which the time of removal of the waste material, washing, and period before beginning the next determination can be materially reduced. This modification consists of the insertion of a steam escape in the stopper of the steam generator flask, and the use of a pinch clamp to shut off alternately the steam going to the vapor vessel and distillation tube, or that escaping through the steam vent. The operation of the apparatus with the steam vent can be illustrated by a description of the washing procedure.

Procedure.—To clean the apparatus (an operation which can be performed before titration in the Jacobs' modification of the Pregl-Parnas-Wagner method), remove the pinch clamp at A_1 and place it at A_2 on the line between the steam generator vessel and the vapor vessel, *T*. This creates a vacuum within three seconds, and the contents of the distillation tube, *D*, are drawn back by suction into the vapor vessel or steam trap. Open the stopcock, S_2, on the small funnel, add 8 to 10 ml. of water, and close the stopcock on the small funnel. The wash water is drawn back promptly by the remaining

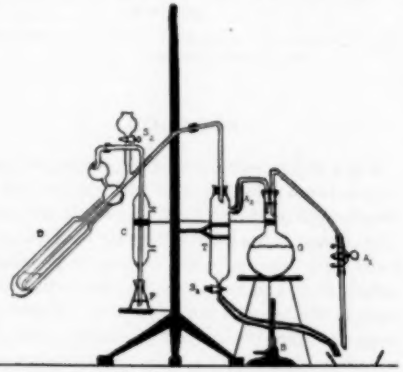

Fig. 1.—Modified Parnas-Wagner apparatus.

vacuum. This may be repeated again. Open stopcock, S_1, on the vapor vessel and allow the waste material to drain. Replace the pinch clamp on the line leading from the vapor escape at A_1, thus permitting the steam to pass into the distillation tube. The apparatus is now ready for the next determination.

It is unnecessary to steam on the first or second washing because the vacuum created is adequate to draw back the rinse water at least twice. Rapid action can be facilitated by moistening the outside of the vapor vessel, *T*.

Pinch clamps may be used at S_1 and S_2 instead of stopcocks, and, conversely, stopcocks may be employed at A_1 and A_2 instead of a pinch clamp, but the use of a pinch clamp in the latter instance permits the almost simultaneous operation of opening the vapor escape and closing the line at A_2.

DISCUSSION

The continued wide use of the Parnas-Wagner apparatus, despite the many other types of apparatus available, even one-piece apparatus, is indicative of its advantages. The modification suggested here retains the apparatus virtually as originally devised, thus retaining its inherent advantages. It also entails no new expense.

The modification suggested saves, depending on the normal operating time for six samples, from one-half to one hour per run. The accu-

* Received May 18, 1950, from the Bureau of Laboratories, Department of Health, City of New York.

racy and precision of the results achieved by use of this apparatus, in faster time, are as good as those obtained with the unmodified apparatus. This is discussed in detail by Jacobs (17).

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Interaction of Streptomycin and *para*-Aminosalicylic Acid on Bacteria. I. Streptomycin-Sensitive *vs.* Streptomycin-Resistant *Escherichia coli*[*,†]

By ROBERTSON PRATT, KSHITISH J. DIVATIA, GRACE GARDNER, and JEAN DUFRENOY

Streptomycin and *para*-aminosalicylic acid (PAS) exert mutually antagonistic biologic effects *in vitro* on a streptomycin-resistant strain of *Escherichia coli*. As the concentration of PAS in the medium is increased, the inhibition due to streptomycin is gradually diminished and ultimately reversed. The organisms are not dependent on streptomycin, except in the presence of relatively high concentrations of PAS. The results, based on *in vitro* experiments, indicate that clinical use of PAS and streptomycin together, after a few doses of streptomycin without PAS have been administered, may be dangerous.

IN THE STRUGGLE for existence, adaptability, which is to say biochemical versatility, is, perhaps, the greatest asset a bacterial cell, a plant, or an animal can possess. Adaptability, in the case of bacteria, often leads to emergence, from strains initially inhibited by chemotherapeutic agents, of strains that are highly resistant to drugs. The ability of some races or strains of bacteria to biochemically circumvent obstacles, such as streptomycin and other antibiotics, that man has learned to put in their way is one of the most troublesome problems encountered in the clinical use of antibiotics. This problem of acquired resistance to antibiotics, especially to streptomycin, has received much attention and has been ably reviewed by several authors (1–4).

In some instances, combined use of two or more antibiotics, or of antibiotics with other antibacterial agents, has been found to retard or to prevent development of resistant strains of bacteria from initially susceptible strains, under conditions that permit resistant strains to develop when only a single antibiotic is used (5–11). PAS has received special attention in this connection because of its usefulness in checking development of streptomycin-resistant races of *Mycobacterium tuberculosis*, when used properly with streptomycin (12–15). PAS not only checks emergence of streptomycin-resistant Mycobacteria when used concomitantly with streptomycin; it also exerts some bacteriostatic action against the tuberculosis organism. Likewise, it inhibits several other species of pathogens, but the concentration required to effect inhibition is many times that required to inhibit *Myco. tuberculosis*.

Reports in the literature are conflicting with regard to the question whether the combined effect of PAS and streptomycin is synergistic, is merely additive, or is no greater than that of streptomycin alone (16–19). This question was partially clarified by Divatia and co-workers (20), who stressed the importance of the relative concentrations of PAS and of streptomycin. Work-

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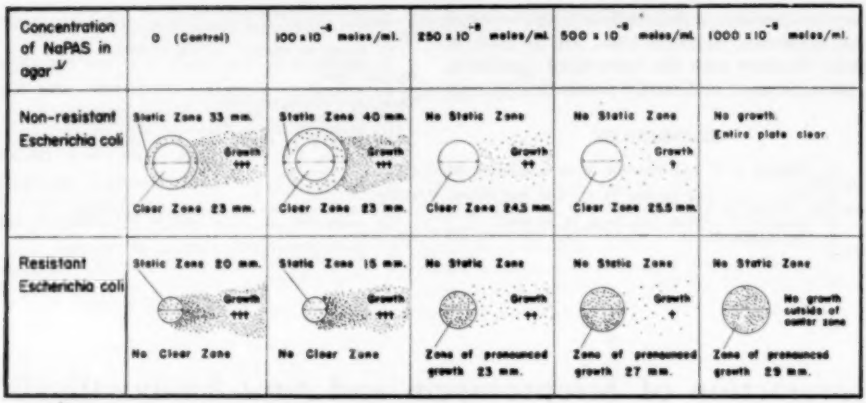
† Thanks are due to the following firms which graciously provided streptomycin and *para*-aminosalicylic acid for these experiments: Barnes-Hind Laboratories, San Francisco, Calif.; Cutter Laboratories, Berkeley, Calif.; Heyden Chemical Corp., Princeton, N. J.; Merck and Co., Rahway, N. J.; and Chas. Pfizer and Co., Brooklyn, N. Y.



[1] Expressed in terms of concentration of para-aminosalicylic acid.

Fig. 1.—Diagrammatic representation of growth responses in cylinder plate tests with a streptomycin-sensitive strain of *Escherichia coli* and of a streptomycin-resistant strain derived from it. Different amounts of PAS were incorporated in the agar. Cylinders (not shown) contained solutions of streptomycin (50,000 μg./ml.) in buffer. Only a small segment of the agar surface, including the area in which growth was affected by streptomycin and part of the adjacent unaffected area, is diagrammed. Density of growth in the background is indicated by a number of + marks.

ing with a streptomycin-sensitive strain of *E. coli*, they observed that, depending on the concentrations employed, PAS enhanced, exerted no effect on, or depressed the antibacterial action of streptomycin. The present paper reports the results of *in vitro* experiments on the growth and inhibition of streptomycin-sensitive and of streptomycin-resistant strains of *E. coli* exposed to PAS and to streptomycin concomitantly. The clinical implications are pointed out.

MATERIALS AND METHODS

Assay plates were prepared according to the conventional cylinder-plate technique. Some plates were prepared with standard nutrient agar (controls); in others the monosodium salt of *para*-aminosalicylic acid (NaPAS) was incorporated in the nutrient agar in quantities calculated to give concentrations of 1×10^{-6} mole to $1{,}000 \times 10^{-6}$ mole per ml. (1.53 to 1,530 μg. per ml.). Concentrations are expressed in terms of *para*-aminosalicylic acid. Each plate was seeded with a 1% suspension from an eighteen-hour broth culture of a strain of *E. coli* originally isolated from feces, or with a suspension of a streptomycin-resistant strain developed from the original sensitive culture by repeated selection and subculturing over a period of four months. Growth of the original culture was prevented completely in nutrient broth containing 7 μg. streptomycin/ml. The resistant strains, developed from this culture, grew in broth containing 50,000 μg./ml. Upon subsequent testing, after successive transfers for an additional four months in broth lacking streptomycin, the resistant strain grew in broth containing 300,000 μg. streptomycin/ml. but failed to grow in broth containing 400,000 μg. streptomycin/ml.

For the tests, solutions of dihydrostreptomycin sulfate were placed in stainless steel cylinders (8 mm. diameter) on the seeded agar surface. Concentrations, in terms of streptomycin base, were 25,000 μg./ml. and 50,000 μg./ml. in different tests. All experiments were repeated several times in quadruplicate.

RESULTS AND DISCUSSION

When seeded test plates were incubated at 37° for eighteen hours, a peculiar difference in reaction of the two strains of *E. coli* was noted. This is best summarized diagrammatically as shown in Fig. 1. The data diagrammed are from experiments with 50,000 μg. streptomycin/ml., but, as shown in Table I, there was no significant difference in the trend of results when streptomycin solutions containing 25,000 μg./ml. were used.

Normally, on test plates containing up to 100×10^{-6} moles (153 mg.) PAS/ml. and seeded with cultures of the nonresistant strain, there were easily discernible three distinct areas representing different degrees of growth. Immediately around the cylinders from which streptomycin was diffusing was a clear zone in which the organisms had failed to grow or, having grown, had been lysed. This area was circumscribed by a band in which growth was clearly evident, although markedly inhibited. In the diagram, this is designated as the static zone to distinguish it from the clear zone where no growth was visible. In the general background of the plate, beyond the area of inhibited growth, the agar was densely crowded with bacterial cells which were not visibly affected by either PAS or streptomycin. On media containing 250 or 500×10^{-6} moles PAS/ml., only two areas were apparent, namely, a clear zone and the general background. In this concentration range, the inhibitory effect of PAS began to become apparent as evidenced by the less

TABLE I.—RESPONSE OF STREPTOMYCIN-RESISTANT AND NONRESISTANT *E. coli* EXPOSED TO STREPTOMYCIN IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF SODIUM *para*-AMINOSALICYLATE

Concn. PAS (Moles/Ml.) in Medium	Diameters of Zones, in Mm. Nonresistant Strain Exposed to Streptomycin 25,000 μg./Ml. Clear	Static	50,000 μg./Ml. Clear	Static	Resistant Strain Exposed to Streptomycin 25,000 μg./Ml.	50,000 μg./Ml.
0	21.5	31	23	23	15.0 static	20.0 static
1×10^{-3}	..	..	..	..	13.4 static	17.1 static
10×10^{-3}	..	..	..	..	13.7 static	18.6 static
100×10^{-3}	22.5	40	23	40	13.5 static	15.0 static
150×10^{-3}	..	..	..	..	18.6 static[a]	24.2 static[a]
200×10^{-3}	..	..	..	..	10.1 clear[b]	11.2 clear[b]
250×10^{-3}	22.5	None	24.5	None	17 enhanced growth	23 enhanced growth
500×10^{-3}	24	None	25.5	None	23 enhanced growth	27 enhanced growth
$1{,}000^{c} \times 10^{-3}$	No zones.		Entire plate clear		29 enhanced growth	29 enhanced growth

[a] Data based on two experiments only. All other figures are averaged from eight experiments performed in quadruplicate.
[b] Clear zones circumscribed by a narrow band of enhanced growth.
[c] At this concentration all plates were entirely clear (no growth) except when seeded with the resistant strain of *E. coli*. In such instances, growth occurred only in an area immediately around the cylinders from which streptomycin was diffusing (see also Fig. 1).

prolific growth of organisms in the background and the absence of a static zone. No growth occurred on media containing $1{,}000 \times 10^{-3}$ moles PAS/ml. whether streptomycin was present or absent.

On plates seeded with the resistant strain, no clear zone appeared around the cylinders containing streptomycin, but some inhibition due to streptomycin was evident on media containing up to 100×10^{-3} moles PAS/ml. (see Fig. 1). It is noteworthy that the inhibition due to streptomycin was less on media containing 100×10^{-3} moles PAS/ml. than when no PAS was present. This suggests that for this resistant strain of *E. coli*, at least in the concentrations used, PAS and streptomycin exert mutually antagonistic actions. This view is confirmed further by consideration of the last three frames showing the response of the resistant organisms. When the concentration of PAS in the medium was 250×10^{-3} moles or more per ml., there was a complete reversal of streptomycin action. Then the resistant *E. coli* grew well only within an area immediately surrounding the cylinder from which the antibiotic was diffusing. It is noteworthy that as the inhibitory effect of PAS became greater with increasing concentrations, the growth-promoting effect of streptomycin increased. These observations were confirmed by treating the plates with triphenyltetrazolium chloride according to the techniques of Pratt and Dufrenoy (1, 21, 22). The tests revealed intense reducing (dehydrogenase?) activity in the areas of pronounced growth. No color change occurred on unseeded plates treated similarly.

The data in Table I (last two columns) show that the turning point between inhibition and stimulation by streptomycin occurred at a PAS concentration of 200×10^{-3} moles/ml. At PAS concentrations below 200×10^{-3} moles/ml., streptomycin inhibited growth of the resistant test organisms, but when higher concentrations were provided, streptomycin stimulated growth. Clearly, the point of balance between these two opposed conditions was reached at a PAS concentration in the neighborhood of 200×10^{-3} moles/ml. Unfortunately, it is not possible to state with any reasonable degree of accuracy the concentration of streptomycin in the agar at a given distance from the cylinder at a given time. Therefore, it is impossible to designate the critical ratio of PAS and streptomycin concentrations in the medium at which the biologic effects of the two drugs exactly balanced each other.

It may be possible to study this problem quantitatively through proper use of aqueous solutions of phloxine. In the medium that was employed, phloxine appeared to specifically stain areas of the plates in which streptomycin was present. When test plates, either seeded or unseeded, on which streptomycin had been permitted to diffuse for several hours, were flooded with a 0.1% aqueous solution of phloxine and then were rinsed with 50% alcohol, a brilliant red zone was seen around each cylinder. The diameters of the stained areas were independent of the concentration of PAS in the medium and were the same whether bacteria were present or absent, but they varied with the concentration of streptomycin in the cylinders. With refinements, this dye may provide a simple and practical reagent for quantitative determinations of streptomycin in nutrient agar.

We were unable to demonstrate a reversal from inhibition to enhancement of growth by streptomycin as the concentration of PAS was increased in broth cultures. The results from plates are difficult to reproduce in broth because of the different conditions that prevail. Probably most important are the differences in aeration and in the complex system comprising the variables, time, concentrations of drugs, and age of the organisms. The importance of threshold and of optimal concentrations of chemicals, and of threshold and optimal reaction times at different stages in the growth of an organism, cannot be overemphasized.

Proper use of phloxine may make it possible to determine the critical streptomycin/PAS ratios at different times on seeded agar plates. If so, by means of appropriate preincubation procedures, similar to those used for studies on the mechanism of antibiotic action (1, 22–25), we expect to demonstrate in broth cultures reversal effects similar to those seen on agar plates.

The concentrations of streptomycin in the experiments reported above considerably exceeded

those normally encountered in the blood or serum of patients under treatment. Therefore, in a second series of experiments, concentrations of streptomycin ranging from high values down to those approaching clinical levels were employed. The results of these experiments are shown in Table II.

TABLE II.—RESPONSE OF STREPTOMYCIN-RESISTANT *E. coli* EXPOSED TO DIFFERENT CONCENTRATIONS OF STREPTOMYCIN IN THE PRESENCE OF SODIUM *para*-AMINOSALICYLATE (100×10^{-6} MOLES/ML.)

Concn. Streptomycin (μg./Ml.) in Cylinders	Zone Characteristics	Diameters of Zones, Mm.[a]
25,000	Static zones only	13.2
2,500	Static zones surrounded by faint ring of enhanced growth	13.0
250	Static zones surrounded by moderately dense ring of enhanced growth	12.4
25	Static zones surrounded by prominent band of enhanced growth 3 mm. wide in some instances	11.0

[a] All zones were static only; no clear zones.

The results from this smaller series of experiments, in which the concentration of streptomycin was varied while the concentration of PAS was uniform, are not as conclusive as in the former series with a larger number of tests, in which streptomycin concentration was uniform and PAS was varied. However, there can be detected a trend from mild inhibition toward enhancement as the concentration of streptomycin placed in the cylinder decreased. Note that at 25,000 μg./ml., only inhibition zones appeared and no enhancement was noted; at 2,500 μg./ml., there was a hint of enhancement around the inhibition zones; as the concentration was lowered successively to 250 and then to 25 μg./ml., the inhibition zones became smaller while the band of enhanced growth became more prominent. This suggests that as some critical ratio of streptomycin to PAS was approached, the inhibitory activity of streptomycin was diminished and, indeed, that the antibiotic began to become stimulatory: in other words, that as this ratio was approached the organisms began to metabolize streptomycin.

IMPLICATIONS AND PROBLEMS FOR FUTURE STUDY

The results reported above suggest strongly that in the streptomycin-resistant strain of *E. coli*, some antagonistic relation exists between streptomycin and PAS. At certain critical concentrations, a similar phenomenon may occur in streptomycin-resistant Mycobacteria. Vennesland, *et al.* (17), observed that inhibition of a streptomycin-sensitive strain of *Mycobacterium tuberculosis* was much enhanced when PAS was added to the antibiotic, but that inhibition of a resistant strain was not increased and, indeed, was diminished somewhat by addition of PAS to streptomycin. Normally, neither chemical is necessary for growth of the strains of *E. coli* used in the present experiments; but when the PAS concentration is sufficiently high to interfere with some process essential for growth, the organisms appear to be able to nullify this effect by metabolizing streptomycin in such a way as to substitute, in part at least, for the process that has been blocked.[1] Only the resistant strain developed the ability to utilize streptomycin. It should be borne in mind that these organisms were not streptomycin-dependent, except in the presence of 200×10^{-6} or more moles of PAS/ml. The organisms grew well in the absence of streptomycin in control media lacking PAS and, indeed, were partially inhibited by streptomycin in such media.

It cannot be argued that the enhancement of growth by streptomycin in the presence of PAS was due to a mere chemical or physical interaction of the two drugs which rendered PAS inactive as an antibacterial agent. If this were true, since the concentration of streptomycin in a given series was uniform, one should expect the zone of enhanced growth surrounding a streptomycin cylinder to become progressively smaller as the concentration of PAS in the medium was increased. Just the reverse was true (Fig. 1, last three frames). Moreover, further evidence of biologically antagonistic action of streptomycin and PAS is seen in Table I and in the first two frames of Fig. 1. When subinhibitory concentrations of PAS were present, i.e., up to 100×10^{-6} moles/ml., streptomycin was less inhibitory than when PAS was absent. That this was not due to inactivation of the antibiotic by PAS was shown by experiments in which solutions of PAS and of streptomycin were mixed and were permitted to diffuse together from the same cylinder on normal media seeded with the resistant organisms. The response of the organisms to these mixtures was the same as to solutions containing the same concentration of streptomycin alone. Further research is required to determine whether this is a true metabolite-antimetabolite relation. The problem is being investigated.

Entirely apart from the fundamental problems of bacterial physiology suggested by these experiments, the results point to potentially important clinical implications. Concomitant use of PAS and streptomycin is receiving increasing favor. When treatment with both drugs is started simultaneously, this appears to be sound practice. There is ample evidence to attest the ability of PAS to prevent or retard development of streptomycin-resistant strains of organisms from initially sensitive ones. But let us consider the case of a patient who has been treated with streptomycin alone. The patient had a low-grade infection which was expected to yield to a short course of streptomycin therapy but the expectation was not fulfilled, and it has become apparent that a longer course of therapy will be required. The physician then might quite logically feel that he should prescribe PAS to be administered along with the streptomycin. This is a potential danger point. The rapidity with which streptomycin resistance can, and often does, develop *in vivo* as well

[1] There was no change in the PAS sensitivity of the organisms as they became resistant to streptomycin. No cross resistance developed. Turbidimetric tests showed that the end point for growth of both organisms in broth lacking streptomycin was between 9.5 and 11.5 mg. PAS/ml. This is supported by the lack of any difference in the appearance of the background growth on test plates. (Compare relative density of growth in areas unaffected by streptomycin, on plates seeded with the streptomycin-sensitive and streptomycin-resistant strains, respectively, as shown in Fig. 1).

as *in vitro* is astonishing. It is quite probable that if an infection treated with streptomycin is not promptly eliminated, some of the surviving organisms will become quite resistant to its action. If the same phenomenon observed in the *in vitro* experiments reported in this paper should occur *in vivo* also, it is quite apparent that, under these conditions, PAS might actually favor growth of the pathogen. We realize that the concentrations used in most of these studies were considerably above the usual clinical blood levels. This does not eliminate the danger, since a trend toward similar effects was observed with considerably lower concentrations (Table II).

Pending confirmation of these results *in vivo*, there seem to be two logical conclusions: in the interest of safety, (*a*) when streptomycin is indicated, routinely administer PAS with the streptomycin *from the beginning of the treatment*, or (*b*) if this has not been done and it is found that a longer course of therapy than initially anticipated is required, discontinue streptomycin and switch to some other antibiotic, the biologic spectrum of which includes the pathogen.

SUMMARY

Using a modified cylinder plate technique, the response of a streptomycin-sensitive strain of *E. coli* to streptomycin with and without the presence of *para*-aminosalicylic acid was compared with the response of a resistant strain derived by subculturing from the original strain.

Growth of the initial strain in broth was prevented by solutions containing 7 μg. of streptomycin/ml.; the resistant strain grew well in solutions containing 300,000 μg./ml. Increased resistance to streptomycin was not accompanied by increase in resistance to PAS; the end point, in broth, was between 9.5 and 11.5 mg. PAS/ml. for the streptomycin-sensitive and the streptomycin-resistant strains.

The original strain yielded the usual inhibition patterns on assay plates, i. e., there were clear zones, circumscribed by static zones of less inhibition, surrounding the cylinders from which the antibiotic (50,000 μg./ml.) had diffused. When the concentration of PAS in the medium was increased to levels at which the PAS itself became inhibitory, the demarcation between the areas inhibited by streptomycin and the background of the plate became less clear. At the highest PAS concentration tested, no growth occurred and the plates were entirely clear.

On plates seeded with the resistant strain, an entirely different response occurred. Such organisms were mildly inhibited by streptomycin (50,000 μg./ml.) in the absence of PAS. When subinhibitory concentrations of PAS were present, the inhibiting effect of streptomycin was diminished as the concentration of PAS was increased, and as the PAS concentration was raised to inhibiting levels, the effect of streptomycin was reversed so that it enhanced the growth of the organisms. When sufficient PAS to completely prevent growth of the bacteria was provided, the organisms grew only in an area into which streptomycin had diffused. Evidence is presented to show that these results were not due to chemical interaction of the two drugs, but were a reflection of biochemical reactions in the cells.

The clinical implications of the observations are discussed.

ADDENDUM

After this manuscript was submitted for publication, clinical papers (26–28) appeared which support the contention that combined PAS-streptomycin therapy should never be started after preliminary treatment with streptomycin alone. Indeed, they go further and show from data obtained in the treatment of human patients that Mycobacteria initially sensitive to PAS may readily develop resistance to this drug. The authors of these papers recommend that when either streptomycin or PAS therapy is indicated, PAS and streptomycin should be used together and that if this has not been done, combined therapy with the two drugs should not be instituted later, whether the drug initially given alone was either streptomycin or PAS.

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A Study of the Effect of Certain Astringents on the Permeability of Frog Skin Using Radioactive Tracer Techniques*

By RUSSELL E. LUX† and JOHN E. CHRISTIAN

Normal frog skin permeability to sodium ions and iodide ions was studied using sodium-24 labeled NaCl and iodine-131 labeled NaI. The effect of certain aluminum salts as astringent agents on normal permeability was determined. A rather unexpected increase in the rate of sodium and iodide ion penetration was noted.

THE ACTION of astringents upon the human skin membranes is not well understood, and the literature on permeability is confused and contradictory. However, it is generally agreed that astringents have a protein-precipitating action which, when applied to a membrane, lessens permeability of that membrane. Goodman and Gilman (1) state that astringent effect is a surface effect, and that the permeability of the cell membrane is greatly reduced.

Previously described methods of evaluating the effect of astringents on membranes include the work of Schuetz (2), who made observations on the suppression of the mucus secretion of frogs after application of the astringent to the breast of the animal, and correlated his findings with astringent activity. Madison and Christian (3) were able to compare the effect of astringents on absorption of sodium ion from the gastrointestinal tract and after subcutaneous injection in the rat by a tracer technique. Govett and de Navarre (4) report evaluations of astringent activity by noting the shrinkage in area of a section of frog skin treated with astringent, and also by noting the decrease in osmotic flow through a portion of frog skin after treating with astringent.

With the recent advances in the utilization of radioactive tracers, new techniques for studying permeability have been devised in this laboratory (5). Astringent effect on permeability was evaluated quantitatively using radioactive tracer techniques by determining the extent of permeability change caused by treating frog membrane with astringent preparations. The findings showed that the astringents used increased, in every case, the permeability of frog membrane to iodide and sodium ions; a finding which was completely unexpected and heretofore unknown.

EXPERIMENTAL

The following procedure was used for studying the effect of aluminum salt astringents on permeability of frog membrane: live immobilized frogs (species *Rana pipiens*) were treated with 250 mg. of an astringent cream, which was prepared by incorporating the desired amount of astringent in a water-washable cream base. (The base was shown to have no effect of itself in membrane permeability.) The astringent cream was brushed on a 6-cm.[2] area of the frog back. A 6-cm.[2] area adjacent to the treated area was left untreated and served as the control. At the end of sixty minutes, the animal was killed, pithed, and the astringent rinsed off the membrane portion that had been treated. The membrane was divided into two similar portions, the treated and the untreated. Using No. 8 cotton thread, the two membrane portions were attached over the ends of 12-mm. glass tubing cut in lengths 15 cm. long, the ends of which had been slightly flared to prevent slippage of the membrane. The membrane was kept moist during the attaching process.

After trimming away excess membrane, 2 ml. of tap water was placed in the upright tubes, and any excess moisture was blotted away from the exposed membrane. The tubes with membranes were then lowered simultaneously into 4 ml. of radioactive iodide[1] (I^{131}) solution having an activity of 200,000 counts per minute per ml.[2] After allowing eighty minutes for equilibrium conditions to be established, 0.1-ml. samples were taken concurrently from the tubes for activity measurements.

It should be noted that excised portions of frog membrane such as this have been shown by many workers to retain "normal" permeability characteristics. In all cases the membranes were oriented so that the morphological inner surface contacted the radioactive solution, and the membrane was extended to a depth of 3 mm. in the radioactive solution. The iodide solutions had a *p*H within the range of 7.4 to 8.3, and the experiments were carried out at room temperature.

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[1] The iodine-131 used in this research was obtained on allocation by the U. S. Atomic Energy Commission.

[2] Assay of radioactive samples was carried out in the following manner: 0.1-ml. aliquots of the solution to be counted were deposited on strips of filter paper 1 x 5 cm. which had been previously impregnated with 10% NaOH. Such strips were then dried thirty minutes under an infrared lamp and, after mounting in cellophane, were counted by placing in the closest proximity, directly under and parallel to, an Eck and Krebs tube. Counts were corrected for background and decay of the isotope, the latter when necessary.

This method was extensively used in testing $AlCl_3$, $Al_2(SO_4)_3$, and aluminum methionate[3] in various percentages. In this work the understanding is implicit that any change in membrane permeability may be followed by comparing the rate of penetration of I^- through the membrane portion being studied with a control membrane portion from the same animal. Thus, any treatment of the membrane which renders it more permeable will be characterized by the more rapid passage of I^- through the treated membrane. The rate of passage of the I^- is determined by radioactive assay.

The results of experiments on a large number of animals showed without exception that the astringents tested in varying concentrations increase permeability of excised frog skin. This was an unexpected result and therefore was investigated in every possible manner. Orientation of the membrane was reversed, methods of application of astringent changed, solutions of astringent instead of creams were used, radioactivity level altered, and, in order to prove that this effect was not peculiar to iodide ion, the permeability of sodium-24 labeled sodium ion, a positively charged ion, was studied. Without qualification, the conclusions that permeability is increased are inescapable. In addition to this, the amount of increase in permeability is proportional to concentration of astringent and increases with time of contact with the ion under study. The amount of increase in permeability of various concentrations of $AlCl_3$ at the time intervals which the treated membranes were in contact with the iodide solution is given in Fig. 1. The plot of results shows that at any time the effect of 5% $AlCl_3$ is approximately twice that of a 2.5% $AlCl_3$, and that 10% $AlCl_3$ gives an effect approximately twice that of 5% $AlCl_3$. Equivalent to the foregoing statement is to say that the slope of 5% $AlCl_3$ *delta* plot is approximately twice that of the 2.5% $AlCl_3$ *delta* plot, etc. Thus, it is possible to calculate the slope of the *delta* plots and to arrive at values characteristic of the percentages of astringents. If one then plots the slopes of the *delta* plots against concentration, a straight line relationship is obtained showing that the effect of the astringent on membrane permeability is directly proportional to the concentration of the astringent used. Similar results were obtained with aluminum methionate and aluminum sulfate. Table I reports the experimental data obtained with 2.5% $AlCl_3$ and is typical of the results obtained with other concentrations and other astringents. Table II summarizes the results obtained with all of the astringents tested.

TABLE I.—THE EFFECT OF 2.5% $AlCl_3$ ON THE PERMEABILITY OF IODIDE ION THROUGH FROG MEMBRANE

Time,[a] Min.	Average Count[b] Untreated Membrane Section	Average Count[b] Treated Membrane Section	Average *Delta* Count
82	97	211	114
122	160	354	194
162	234	519	285
202	311	691	380

[a] Time the membranes were in contact with the iodide solution.
[b] Average count per 0.1-ml. aliquot from inner solution of 11 different membranes at each time interval.

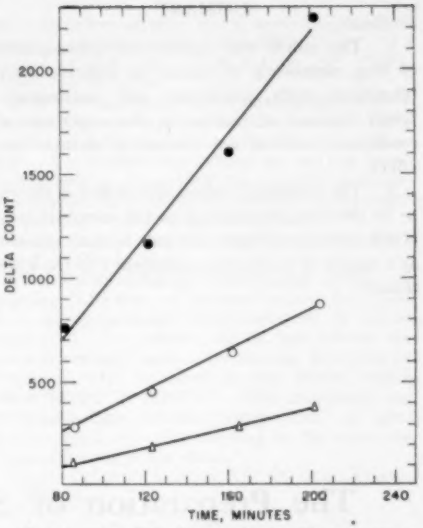

Fig. 1.—Average *delta* plots for three percentages of $AlCl_3$. ●, 10% $AlCl_3$; ○, 5% $AlCl_3$; Δ, 2.5% $AlCl_3$. *Delta* count = difference between activities of inner solutions in the case of treated and untreated membranes. Time = time membranes were in contact with iodide solution.

TABLE II.—THE EFFECT OF ASTRINGENTS ON THE PERMEABILITY OF IODIDE ION THROUGH FROG MEMBRANE

Astringent	Strength, %	No. of Animals[a]	Average *Delta* Count,[b] Min.[c] 82	122	162	202	Mean Slope
$AlCl_3$	2.5	11	114	194	285	380	2.624
$AlCl_3$	5.0	12	262	454	646	881	5.393
$AlCl_3$	10.0	11	746	1166	1662	2250	12.39
$Al_2[CH_2(SO_3)_2]_3$	2.5	10	95	174	313	493	2.222
$Al_2[CH_2(SO_3)_2]_3$	10.0	9	648	876	1261	1749	9.118
$Al_2(SO_4)_3$	1.5	11	89	117	258	409	2.961
$Al_2(SO_4)_3$	2.5	11	81	172	320	455	3.342

[a] Number of animals at each time interval
[b] Is the average difference between the activity of the inner solutions in the case of the treated and untreated membranes.
[c] Time the membranes were in contact with the iodide solution.

[3] Synthesized in the laboratories of the Purdue University School of Pharmacy.

SUMMARY

1. The iodide and sodium ion permeability of frog membrane is shown to increase after treatment with aluminum salt astringents. Every manner of change in the experimental conditions, without any exception, showed this effect.

2. The increased permeability effect is shown to be directly proportional to the concentration of the astringents used, and may be thus utilized as a means of evaluating astringency of the salts tested.

3. The technique developed may be applicable to comparing the effectiveness of antiperspirant preparations and may be extended to the study of the effect of a variety of pharmaceutical preparations on skin and other membranes.

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The Preparation of Schiff Bases from Some Sulfonamides and Aromatic Aldehydes*

By RAYMOND N. CASTLE

The preparation of eight new Schiff bases derived from therapeutically active sulfonamides is reported.

THE REACTION of aromatic aldehydes with aromatic primary amines to produce Schiff bases is well known. Many of the therapeutically useful sulfonamides contain a free primary amino group, thus it appeared of interest to prepare some Schiff bases from some of the sulfonamides and aromatic aldehydes.

Several investigators (1–3) have prepared Schiff bases from sulfanilamide. Schonle (4) prepared therapeutic sugar derivatives of sulfapyridine for the purpose of increased solubility of the sulfonamide. These were described as Schiff base-type compounds. A series of Schiff bases have been prepared for a number of sulfonamides with both salicylaldehyde and 2-hydroxy-3-methoxybenzaldehyde by the author (5). The present paper is a continuation of the above study. The previously published series of compounds was prepared for purposes of identification via the optical crystallographic properties, while the present series was prepared as potentially active bacteriostatic agents.

* Received September 20, 1950, from the Laboratory of Pharmaceutical Chemistry, College of Pharmacy, The University of New Mexico, Albuquerque.

It is well known that Schiff bases are hydrolyzed with ease into the original aldehyde and amine. It appeared then that perhaps these compounds would liberate the active sulfonamide over a period of time, producing higher sustained blood levels. However, the present paper will deal only with the methods of preparation of these compounds and their physical and chemical properties.

Eight Schiff bases have been prepared.

EXPERIMENTAL

The compounds prepared were dried *in vacuo* over phosphorus pentoxide at 95° for from two to three hours prior to their analysis.

N^4-*p*-Chlorobenzylidene Sulfaguanidine (I).—To a hot ethanolic solution of 4.28 Gm. of sulfaguanidine was added 2.81 Gm. of *p*-chlorobenzaldehyde. The solution was refluxed for one and one-half hours. On cooling, 2.20 Gm. of pale yellow needles of N^4-*p*-chlorobenzylidene sulfaguanidine separated. The compound melted at 237.5 to 238.5° after recrystallization from butyl Cellosolve.

Anal.—Calcd. for $C_{14}H_{13}ClN_4O_2S$: N, 16.63. Found: 16.42, 16.43.

N^4-*p*-Methoxybenzylidene Sulfaguanidine (II).—To a hot absolute ethanolic solution of 2.14 Gm. of sulfaguanidine was added 1.36 Gm. of anisaldehyde. The solution was refluxed for one hour. On standing, 2.81 Gm. of pale yellow plates separated, which upon recrystallization from butyl Cellosolve melted at 240.5 to 241°.

Anal.—Calcd. for $C_{15}H_{16}N_4O_5S$: N, 16.86. Found: 16.72, 16.76.

N⁴-*m*-Nitrobenzylidene Sulfaguanidine (III).—To 1.07 Gm. of sulfaguanidine in boiling absolute ethanol was added 0.75 Gm. of *m*-nitrobenzaldehyde. The yellow solution was refluxed one hour. On cooling, 1.37 Gm. of N⁴-*m*-nitrobenzylidene sulfaguanidine separated as a pale yellow microcrystalline powder which, after washing with cold ethanol, melted at 221–224°.

Anal.—Calcd. for $C_{14}H_{13}N_5O_4S$: N, 20.17. Found: 19.75, 19.78.

N⁴-*o*-Chlorobenzylidene Sulfaguanidine (IV).—To 2.14 Gm. of sulfaguanidine in absolute ethanol was added 1.40 Gm. of *o*-chlorobenzaldehyde. The yellow solution was refluxed for one-half hour. As the solution cooled, 2.42 Gm. of N⁴-*o*-chlorobenzylidene sulfaguanidine separated as an almost colorless microcrystalline powder. After repeated washing with cold ethanol, compound IV melted sharply at 219.5°.

Anal.—Calcd. for $C_{14}H_{13}ClN_4O_2S$: N, 16.63. Found: 16.95, 16.67.

N⁴-*p*-Methoxybenzylidene Sulfacetimide (V).—To 2.12 Gm. of sulfacetimide in absolute ethanol was added 1.36 Gm. of anisaldehyde. The solution was refluxed two and one-fourth hours. On standing and cooling, the compound could not be induced to crystallize. However, crystallization was accomplished by evaporating the solvent and cooling in the refrigerator, whereupon 2.50 Gm. of hard, nearly colorless crystals separated which melted at 200.5 to 203.5° after recrystallization from ethanol.

Anal.—Calcd. for $C_{16}H_{16}N_2O_4S$: N, 8.43. Found: 8.28, 8.32.

N⁴-*m*-Nitrobenzylidene Sulfacetimide (VI).—To 1.06 Gm. of sulfacetimide dissolved in boiling absolute ethanol was added 0.75 Gm. of *m*-nitrobenzaldehyde in absolute ethanol. The solution was refluxed for two-thirds of an hour and some of the solvent evaporated. On standing in the refrigerator, 1.49 Gm. of compound VI separated as very fine pale yellow crystals which, upon recrystallization from ethanol, melted at 218–220°.

Anal.—Calcd. for $C_{15}H_{13}N_5O_6S$: N, 12.10. Found: 11.78, 11.74.

N⁴-*p*-Methoxybenzylidene Sulfathiazole (VII).—To 2.54 Gm. of sulfathiazole dissolved in boiling absolute ethanol was added 1.36 Gm. of anisaldehyde. The solution was refluxed for two and three-fourths hours. On cooling the solution, 2.70 Gm. of pale yellow crystals separated which, upon washing with cold ethanol, melted at 198–201°.

Anal.—Calcd. for $C_{17}H_{15}N_3O_3S_2$: N, 11.26. Found: 10.91, 10.96.

N⁴-*m*-Nitrobenzylidene Sulfadiazine (VIII).—A quantity (1.25 Gm.) of sulfadiazine and 0.75 Gm. of *m*-nitrobenzaldehyde were dissolved in glacial acetic acid. The yellow solution was refluxed for one and one-half hours. On cooling, 1.50 Gm. of compound VIII separated as fine yellow tablets which melted at 218–220°. This compound, on standing in light, exhibits phototropism. In light, it turns brick red. On standing in the dark, the compound turns yellow again.

Anal.—Calcd. for $C_{17}H_{13}N_5O_4S$: N, 18.27. Found: 17.78, 17.75.

SUMMARY

Eight Schiff bases have been prepared from some therapeutically active sulfonamides and aromatic amines.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Allyl butyl barbituric acid
Anthrylmercapturic acid
Carbonic anhydrase
Cardol
1,2-Chrysoquinone
Coenzyme 11
cis-β-Decalone
Dicarbazyl
1,1-Dimethylethylene oxide
Ethenyl tricarboxylic acid
4-(N-Ethylamino)azobenzene
s-Di-2-naphthyl semicarbazide
1,3-Di-1-naphthylguanidine
Heptadecyl alcohol
trans-Hexahydro-*p*-toluic acid
1,2-Naphthoquinone-4-sulfonic acid
N-Phenyl-N-benzyl urethane
1-Phenyl indane
Pyrene-3,8-quinone
β-Sulfo propionic acid

Polarographic Studies of Some Antihistamine Compounds and Some Official Iron Preparations*

By L. E. BINGENHEIMER, Jr.,† and JOHN E. CHRISTIAN

A group of antihistamine compounds has been studied, and the compounds have been found polarographically reducible. The polarographic reactions of a group of official iron preparations have also been studied, and a method for the polarographic determination of iron in these preparations has been developed. The method has as its basis the formation of a citrate-ferric ion complex and the comparison of polarographic wave heights of the sample and a similar reference solution of known iron content.

A NUMBER of antihistaminic agents have appeared on the market in recent years, most of which combine marked but fleeting palliative powers with rather low toxicity. Little has been published concerning assays of these preparations, but communications from the manufacturers indicate that, to date, spectrophotometric methods are used in most cases, along with a few colorimetric and gravimetric methods. There is no record of polarographic determination of the antihistamine compounds, although some related compounds have been subjected to polarographic examination. The literature indicates the possibility of two centers of polarographic activity in most of the antihistamine compounds, the pyridine or similar nitrogen heterocycle nucleus and the tertiary or quaternary amine group (1, 2).

The official preparations of ferric iron are all assayed volumetrically, generally by the titration of iodine liberated by the oxidizing action of the ferric ion. Almost all of the official ferrous preparations are assayed volumetrically against ceric sulfate. In one case, that of ferrous iodide syrup, the iron content is determined indirectly by argentimetric titration of the anion.

The bulk of the literature on polarographic properties of iron deals with the assay of iron and steel alloys (3, 4). Various complexes of iron have been suggested for polarographic study. Sanigar (5) investigated the ferrocyanide, Stackelberg and Freyhold (6) the oxalate and fluoride complexes, and Komarek (7) the ferric-mannitol complex. Lingane (8) reviewed the action of oxalate, citrate, and tartrate complexes of iron. The problem faced in polarographic assay of the pharmaceutical iron preparations differs from the other applications in the variety of forms of iron encountered and the types of material which may interfere with the determination. Leonhardt (9) and Bowman, *et al.*, (10), report the use of the polarograph to detect the presence of small amounts of heavy metals in pharmaceutical products. Kamegai (11) mentions the use of the polarograph for determination of iron in biological materials. There is no record of the application of the polarographic method to the determination of the pharmaceutical preparations of iron.

EXPERIMENTAL

Apparatus.—The instrument used was the Sargent Model XX visible recording polarograph. The electrolysis cell of Lingane and Laitinen (12) was used, and temperature control within the range of 25° ± 1° was effected by the use of a large water bath containing a coil of soft copper tubing connected to a constant-temperature water circulator. The oxygen maximum was suppressed by the addition of a small amount of gelatin to the solution, followed by bubbling with nitrogen. The height of the mercury column and the degree of damping were maintained constant, and concentrations of solutions were adjusted to permit the use of sensitivities in the second range of the instrument.

The Antihistamine Compounds.—The group of antihistamine compounds studied included Anthallan, Antistine, Benadryl, Decapryn, Histadyl, Neo-Antergan, Neohetramine, Pyribenzamine, and Trimeton. All of these compounds contain one or more tertiary amine groups, and in most of them there is a nitrogen heterocycle group. The following salts were used as supporting electrolytes in the course of the investigation: ammonium chloride, 0.05%, 0.1%, and 0.1 *M*; potassium chloride, 0.1 *M*; tetramethylammonium bromide, 0.1 *M*; and tetraethylammonium bromide, 0.1 *M*. Ammonium chloride and potassium chloride were reduced at lower potentials than were the antihistamines, and thus were unsatisfactory as supporting electrolytes. The quaternary salts were found to be satisfactory only after three recrystallizations from alcohol and water.

The reactions of all the antihistamine compounds studied were similar. The reduction curves which were obtained started in a very regular manner, but in each case they became irregular before the

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peak was reached, and wave heights varied greatly. The size of the curves bore only a slight relationship to concentration, and the shape varied markedly with small changes in concentration. Curves were not found to be reproducible.

The Official Iron Preparations.—The polarographic reactions of ferric chloride and ferrous sulfate in concentrations from 10^{-3} *M* to 10^{-2} *M* were studied in the following supporting electrolytes: 0.5 *M* sodium citrate, 0.5 *M* sodium tartrate, 0.5 *M* ammonium tartrate, and 0.2 *M* sodium oxalate. As a general rule, wave height was found to be proportional to concentration, although results varied with change in the supporting electrolyte. Sodium citrate was found to be the most satisfactory supporting electrolyte, giving good, easily measurable waves and offering a good buffering action.

The wave height of the complex formed by the ferric ion in 0.5 *M* sodium citrate solution was found to be unchanged by variations in *p*H within the range 4.5 to 6.5. The wave height of ferrous ion decreased slowly over the *p*H range 2.0 to 7.0. Air oxidation of ferrous ion interfered with the determination of the ion; for this reason, ferrous salts were completely oxidized to the ferric state and the ferric ion content was determined.

Salts which exist in stable complex formations are not completely dissociated in aqueous solution; therefore, it was necessary to use heat and to increase the acidity of the solutions to liberate ferric ion, which then forms the desired citrate complex for the analysis.

The polarographic method was applied to 23 of the 38 official salts and preparations of iron, representing all the types of pharmaceutical preparations in which iron is official. The general method was as follows: the iron was obtained in solution as the ferric ion, and polarographic solutions containing about 1 mg. of iron per cc. in 0.5 *M* sodium citrate as supporting electrolyte and complexing agent, and 0.005% gelatin for suppression of the oxygen maximum, were prepared and analyzed. The iron content was determined by comparison of the wave height with the wave height of similar solutions containing known amounts of iron. Results of the polarographic assay were compared with the results of the official assay, or of modifications of the official assay which were necessary either for purposes of comparison or because of apparent inaccuracies encountered in the official assay.

The assay of ferric ammonium citrate supplies an example of special treatment of the polarographic sample, as well as of modification of the official assay method. Preliminary iodometric assays of ferric ammonium citrate by the official method gave unsatisfactory results. Following the completion of the iodine titration a brown color persisted in the solution, indicating incomplete reaction. Results appeared to be rather low (16.89% Fe). The official method was modified as follows: approximately 4 Gm. of ferric ammonium citrate, accurately weighed, was dissolved in 50 cc. of distilled water in a beaker, and 10 cc. of hydrochloric acid was added. The solution was boiled for ten minutes, and allowed to cool. The solution was then transferred quantitatively to a volumetric flask and the volume was made up to 200 cc. with distilled water. Exact 25-cc. aliquots were then transferred to iodine flasks, and titrated as in the official assay. A relative increase of about 3% in experimental results, to 17.24% Fe, was observed, and there was no residual brown color following the titration.

Four samples of ferric ammonium citrate were prepared for polarographic determination as follows: 200 cc. of solution, containing approximately 4 Gm. of ferric ammonium citrate, accurately weighed, was prepared by the above method, and 25-cc. aliquots were transferred to 100-cc. volumetric flasks, each of which contained 50 cc. of molar sodium citrate solution, 2 cc. of 0.25% gelatin solution, and 1 cc. of hydrochloric acid. Distilled water was added to give a final volume of 100 cc. (The final solution, containing the above proportions of sodium citrate, gelatin, and hydrochloric acid, is referred to hereinafter as the "polarographic solution.")

From 15 to 20 cc. of each polarographic solution was placed in a clean electrolysis cell, nitrogen gas was passed through the solution for ten minutes, and three polarograms were recorded for each solution. Instrument settings were as follows: initial potential, 0 v.; span potential, 3 v.; sensitivity 2–3 (range 2, vernier 3); damping, 3. The iron content was calculated by comparing the average wave height of the samples with the average wave height of four samples of a ferric chloride solution of known concentration electrolyzed under the same conditions:

Average wave height of unknown solutions, mm	242
Average wave height of ferric chloride solution, mm	232
Fe content of ferric chloride solution, mg./cc.	3.326

$$\frac{242}{232} \times 3.326 = 3.469 \text{ mg. Fe/cc.}$$

$$\frac{3.469 \text{ mg./cc.} \times 200 \text{ cc.}}{4027.0 \text{ mg. (weight of sample)}} \times 100 = 17.23\% \text{ Fe}$$

This value represents a deviation of less than 0.1% from the result of the modification of the U. S. P. method.

Table I gives the results of the polarographic assay of the pharmaceutical iron preparations used. All analyses were carried out as described for ferric ammonium citrate, with the exception of the treatment of samples as noted.

Methods

Method A.—Dissolve sample in 50 cc. distilled water, add 10 cc. hydrochloric acid, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask, make up to 200 cc. with distilled water.

Method B.—Dissolve sample in distilled water to make exactly 200 cc. To aliquot of this solution add 1 cc. hydrochloric acid and 10 cc. hydrogen peroxide solution, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask for preparation of polarographic solution.

Method C.—Add 10 cc. diluted sulfuric acid to powdered sample, and 40 cc. distilled water, allow to stand fifteen minutes, filter quantitatively, add 1 cc. nitric acid, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask for preparation of polarographic solution. Hydrogen peroxide did not satisfactorily oxidize this preparation, presumably because of the presence of organic matter. The nitric acid gave a nitrate wave on the

TABLE I.—METHODS AND RESULTS OF POLAROGRAPHIC ASSAY OF OFFICIAL IRON PREPARATIONS

Preparation	Sample	Method of Sample Treatment	Aliquot, Cc.	Final Volume[a]	Polarographic Assay, %[b]	Official Assay, %[b]	Deviation, %
U. S. P.							
Ferric ammonium citrate	4.0 Gm.	Method A	25	100	17.23	17.24[c]	0.06
Ferrous sulfate	4.0 Gm.	Method B	25	100	20.04	20.06[d]	0.10
Ferrous sulfate tablets	1.0 Gm.	Method C	..	200	59.78	59.85	0.12
N. F.							
Green ferric ammonium citrate	6.5 Gm.	Method D	10	100	14.66	14.67	0.07
Ferric cacodylate ampuls	10 cc.	Method E	..	100	0.5334	0.5331[e]	0.06
Ferric chloride solution	2.0 Gm.	Method F	10	50	13.06	13.07	0.08
Ferric chloride tincture	10 cc.	Method G	10	50	4.155	4.149	0.14
Ferric citrochloride tincture	10 cc.	Method G	10	50	4.790	4.795	0.10
Ferric glycerophosphate	1.0 Gm.	Method H	25	100	15.82	15.80[f]	0.13
Ferric hypophosphite	1.0 Gm.	Method I	25	100	21.97	21.96	0.05
Red ferric oxide	1.5 Gm.	Method J	25	100	99.06	99.15	0.09
Yellow ferric oxide	1.5 Gm.	Method J	25	100	86.54	86.61	0.08
Soluble ferric phosphate	2.0 Gm.	Method K	25	100	13.88	13.86	0.14
Ferric subsulfate solution	10.0 cc.	Method L	25	100	21.22	21.26	0.19
Ferric sulfate solution	2.7 Gm.	Method F	25	100	10.45	10.47	0.19
Beef, iron and wine	10.0 cc.	Method M	..	100	0.8216	0.8246	0.37
Iron and ammonium acetate solution	25.0 cc.	Method N	..	50	0.1735	0.1739	0.23
Peptonized iron and manganese solution	25.0 cc.	Method N	..	100	0.2834	0.2846	0.42
Ferrous carbonate pills	1.5 Gm.	Method O	25	100	18.87	18.90[g]	0.16
Saccharated ferrous carbonate	2.0 Gm.	Method P	25	100	19.62	19.64	0.10
Ferrous gluconate	1.5 Gm.	Method P	25	100	11.44	11.45	0.09
Ferrous iodide syrup	10.0 cc.	Method Q	25	100	7.181	7.201	0.28
Reduced iron capsules	0.4 Gm.	Method R	25	100	90.40	90.49	0.10

[a] Solution containing in each 100 parts 50 parts molar sodium citrate solution, and 2 parts 0.25% gelatin solution.
[b] Averages of at least three samples prepared independently.
[c] For modification of official assay see text of paper.
[d] Ceric ammonium sulfate was substituted for potassium permanganate in the assay of ferrous sulfate, and for ceric sulfate in the assays of ferrous sulfate tablets, ferrous carbonate pills, saccharated ferrous carbonate, ferrous gluconate, and reduced iron.
[e] The National Formulary gives no assay for iron in ferric cacodylate ampuls. The assay for iron in ferric cacodylate was adapted to assay of the ampuls by taking an accurately measured sample of the ampul solution to contain about 0.5 Gm. of ferric cacodylate.
[f] Drying the sample at 130° caused lumping and partial decomposition. When the drying step was omitted from the official assay method, it was found that the addition of sodium bicarbonate, as directed, reduced the acidity of the solution to be titrated to a point where the liberation of iodine was not complete within the time specified. Uniform, nonheated samples were used and the sodium bicarbonate was omitted.
[g] Thirty pills were accurately weighed, crushed, and powdered, and accurately weighed samples taken.

polarogram, which made accurate measurement difficult but not impossible.

Method D.—Dissolve in distilled water to make exactly 100 cc. Add 2 cc. hydrochloric acid to aliquot, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask for preparation of polarographic solution.

Method E.—Add 2 cc. hydrochloric acid, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask for preparation of polarographic solution.

Method F.—Dilute to exactly 100 cc. with distilled water.

Method G.—Dilute to exactly 100 cc. with distilled water. Boil aliquot ten minutes, allow to cool; transfer quantitatively to volumetric flask for preparation of polarographic solution. Preliminary tests, which omitted the boiling step, gave low results, probably because of the presence of alcohol. Perrachio and Meloche (13) found that when alcohol-water mixtures were used as solvents, lower diffusion currents were observed than with water alone. When the alcohol was removed by boiling, results were satisfactory.

Method H.—Dissolve in 25 cc. distilled water, add 5 cc. hydrochloric acid, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask, make up to 100 cc. with distilled water.

Method I.—Dissolve in 10 cc. nitrohydrochloric acid, evaporate to dryness, add 5 cc. hydrochloric acid, evaporate to dryness again, dissolve residue in 5 cc. hydrochloric acid and 25 cc. distilled water, transfer quantitatively to volumetric flask, make up to 100 cc. with distilled water.

Method J.—Dissolve in 25 cc. warm hydrochloric acid, add 10 cc. hydrogen peroxide solution, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask, make up to 250 cc. with distilled water.

Method K.—Dissolve in 5 cc. hydrochloric acid, make up to 100 cc. with distilled water.

Method L.—Dilute to 100 cc. with distilled water, transfer 25 cc. to another volumetric flask and make up to 200 cc.

Method M.—Evaporate to dryness, ignite strongly to destroy carbonaceous matter, add 10 cc. hydrochloric acid and 10 cc. hydrogen peroxide solution, evaporate to dryness; dissolve residue in 5 cc. hydrochloric acid and 25 cc. distilled water, transfer quantitatively to volumetric flask for preparation of polarographic solution. The large amount of organic matter present in this preparation made direct polarographic analysis impossible, and also prevented quantitative precipitation of the iron as ferric hydroxide, so that ignition was necessary. Such was also the case with iron and ammonium acetate solution, peptonized iron and manganese solution, and ferrous iodide syrup.

Method N.—Same as method M except that the final residue is dissolved in 5 cc. hydrochloric acid and 5 cc. distilled water.

Method O.—Add 5 cc. hydrochloric acid and 25 cc. distilled water, allow to stand fifteen minutes, filter quantitatively into volumetric flask, make up to 50 cc. with distilled water; add 1 cc. nitric acid to aliquot, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask for preparation of polarographic solution.

Method P.—Dissolve in 10 cc. diluted sulfuric acid and 10 cc. distilled water, add 1 cc. nitric acid, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask, make up to 50 cc. with distilled water.

Method Q.—Same as method M except final residue dissolved in 5 cc. hydrochloric acid and 25 cc. distilled water, transferred quantitatively to volumetric flask and made up to 50 cc. with distilled water.

Method R.—Dissolve in 5 cc. hydrochloric acid, add 10 cc. hydrogen peroxide solution, boil ten minutes, allow to cool; transfer quantitatively to volumetric flask and make up to 100 cc. with distilled water.

SUMMARY AND CONCLUSIONS

1. A group of the antihistamine compounds was studied and found to be polarographically reducible.

2. Reduction waves of the antihistamines were found to be nonreproducible under the conditions of these experiments.

3. Application of the polarographic method to 23 widely varying official forms of iron indicated that the polarographic method is satisfactory for the assay of most of the simple or moderately complex salts and preparations.

4. In the cases of ferric ammonium citrate and ferric glycerophosphate the polarographic method appears to be more accurate than the official assays, the conditions of which do not favor complete reaction and thus tend toward erroneous results.

5. In the case of preparations containing large amounts of organic matter, the difficulty in separating the iron from materials which would interfere with polarographic determination, as well as the relatively low degree of accuracy, prevent the acceptance of the polarographic method as a completely practical substitute for the official assays of these preparations.

6. The advantages of the polarographic method in the assay of the official iron preparations lie in the adaptability of the method to analytical control involving the analysis of large numbers of samples, the elimination of unstable reagent solutions which require frequent re-standardization, and the permanent record afforded by the polarogram.

7. The chief disadvantage of the polarographic method for the assay of official iron preparations lies in the necessity of obtaining the iron in the completely ionized ferric state, sometimes requiring drastic treatment.

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Sabadilla Alkaloids. III. Chromatographic Separation of the Water-Soluble Fraction. Isolation of a New Crystalline Alkaloid, Sabatine*,†

By ARNOLD J. HENNIG,‡ TAKERU HIGUCHI, and LLOYD M. PARKS

By partition chromatography on a buffered silicic acid column, evidence has been obtained for the presence of at least five additional alkaloids in the water-soluble portion, crude fraction D (see paper I in this series) of sabadilla alkaloids. One of the new alkaloids has been obtained in crystalline state and given the name "sabatine." Upon hydrolysis it gives rise to a new alkanolamine, which has been given the name "sabine." Some of its properties and the results of preliminary pharmacological tests are reported.

In previous reports there was described a method of extraction and partial separation of the total alkaloids of sabadilla seed into four crude fractions, designated A, B, C, and D (1), and the isolation, by fractional solution and fractional crystallization, of cevadine and veratridine from crude fraction A. The present report describes further chromatographic studies on crude fraction D, by which means it was fractionated into its alkaloidal components.

Since the known sabadilla alkaloids, i. e., cevadine, veratridine, and cevine, exhibit characteristic absorption spectra in the ultraviolet region, the absorption spectra of the four crude fractions A, B, C, and D were determined and are shown in Fig. 1.[1] Comparison of the absorption spectra of these fractions with those of the known alkaloids (2) led to the conclusion that crude fraction D was the most likely to contain new alkaloids. Since crude fractions A, B, and C exhibit similar absorption characteristics, namely, maxima at 262 and 292 mμ, it may be concluded that veratridine, with maxima at 262 and 292 mμ, is a major component of these three fractions. Also, since crude fraction D shows the absorption characteristics of veratridine only very slightly, that alkaloid may be considered absent from this fraction. Cevadine also is probably absent from this fraction since its absorption becomes very high at approximately 243 mμ.

Crude fraction D, according to its method of separation (1), is composed of the water-soluble alkaloids of sabadilla. On the basis of solubility it might be expected that cevine, which is insoluble in water, would also be absent from this fraction. Thus, on the basis of ultraviolet absorption and solubility data, it might be concluded that the three known alkaloids of sabadilla are absent from crude fraction D. Since this fraction seemed the most likely to contain new alkaloids, it was chosen for further investigation.

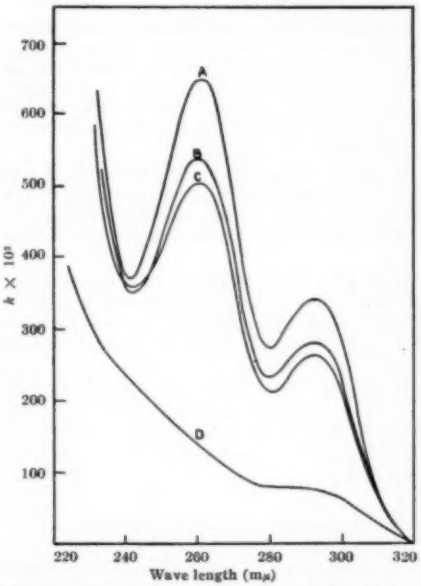

Fig. 1.—The ultraviolet absorption spectra of fractions A, B, C, and D of the sabadilla alkaloids.

Since preliminary work (2) had indicated that adsorption chromatography might prove useful in the separation of new alkaloids, this procedure was explored with crude fraction D. When it became apparent that it did not produce any reasonably clear-cut separations, the more recent procedure of partition chromatography,

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[1] All spectrophotometric data were obtained with a Beckman Model DU Quartz Spectrophotometer. The solvent was absolute alcohol.

originated by Martin and Synge (3) was used. The latter procedure has proved especially useful in the separation of amino acids, and Evans and Partridge have demonstrated its effectiveness in the separation of hyoscine and hyoscyamine (4), and in the investigation of the constituents of *Datura ferox* (5).

EXPERIMENTAL

Adsorption Chromatography.—Since no data were available on the adsorption characteristics of the alkaloids, various adsorbents, ranging from weak to strong in nature, were tried. Powdered sugar gave only weak adsorption and no separation of components, while calcium carbonate showed slightly more adsorption but little or no separation.

Magnesium Oxide.[2]—A slurry of this adsorbent with chloroform was prepared and poured into a 3.4 x 23-cm. tube, fitted with a sintered glass disk which had been previously layered with one-eighth inch of Filter Cel. The column thus prepared was 9 cm. in height and had a deep violet appearance under ultraviolet light. It was fitted to a bell jar equipped for suction. A flow rate of effluent of 1 cc. per minute was maintained, during development and elution of the chromatogram, by the use of slight suction.

A sample of crude fraction D (567 mg.), dissolved in a minimum amount of chloroform, was adsorbed on the column and developed with 100 cc. of chloroform. At this point at least six different bands could be observed under ultraviolet light, as shown in Fig. 2. The most rapidly moving band was sharply defined, bright yellow (I), and about 25 mm. from the bottom. Above this band were two more yellow bands (II and III) with well-defined outer edges but with a yellow diffuse area separating them. Above these bands was a 22-mm. area (III-a) of the deep violet color of the original magnesium oxide. The remaining three bands, dark blue (IV), light blue (V), and yellowish-green (VI), were each about 1 mm. wide and separated from one another by approximately the same distance.

Three large fractions of chloroform eluate were collected. The first one, 200 cc., apparently contained all of band I, and an alkaloidal test on the eluate at this point was very faint. After the second and third fractions, 140 cc. each, were collected, alkaloid tests were negative. Elution of the chromatogram was discontinued at this point; the excess chloroform was removed by suction, the column extruded from the tube, cut lengthwise, and examined under ultraviolet light. The lower half was now light yellow, apparently made up of bands II and III with the diffuse area between them. The upper half appeared light blue, with the exception of an 8-mm. portion at the top, which contained bands IV, V, and VI so close together that their separation was impossible.

The column was now cut crosswise into three portions, yellow, light blue, and the top 8-mm. portion, and the portions extracted separately with warm methyl alcohol, either by successive washings or by Soxhlet extraction. The solvents were distilled off, the residues taken up in 5% H_2SO_4, made alkaline with ammonia, and alkaloidal material shaken out with chloroform. Table I shows the amounts of alkaloidal material recovered from the various elutions and washings of the column; it represents a recovery of 63.5%.

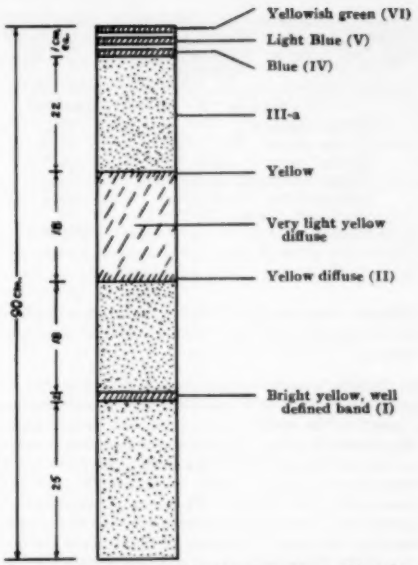

Fig. 2.—Magnesium oxide chromatogram of fraction D of the sabadilla alkaloids under ultraviolet light.

Aluminum Oxide.[3]—In all experiments with this adsorbent the alkaloidal mixture was so strongly adsorbed at the top of the column that it could not be moved by chloroform, and required 50–100% alcohol to cause elution. With this solvent a large proportion of the mixture (approximately 75%) was eluted at one time while the remainder could not be moved except by the addition of 0.5 to 1% ammonia to the solvent.

PARTITION CHROMATOGRAPHY

Stationary Phase.—Buffered silicic acid was found to give the most suitable separation. Of four brands of silicic acid tried, the one obtained from Mallinckrodt, especially prepared for chromatographic analysis, appeared to have a finer particle size and gave the best results. The buffer solution was prepared from equal quantities of 0.5 *M* monosodium phosphate and 0.5 *M* disodium phosphate. The *p*H of this buffer was adjusted by addition of a saturated solution of sodium hydroxide or 85% phosphoric acid until the desired *p*H was reached. The buffer solutions used in this work were adjusted to *p*H 8.5. This was found to be the most satisfactory from the standpoint of separation of components and recovery of material from the column. Shifting the *p*H was used in numerous instances to

[2] Merck reagent, heavy, heated for five hours to about 600°.

[3] Aluminum Company of America, Grade F-1, mesh—100.

TABLE I.—ALKALOIDAL MATERIAL RECOVERED FROM MAGNESIUM OXIDE CHROMATOGRAM OF 567 Mg. of CRUDE FRACTION D OF SABADILLA ALKALOIDS

Fraction	Weight (Mg.)
200 cc. eluate	76.7
140 cc. eluate	34.6
140 cc. eluate	5.3
Yellow cut (bands II, III)	105.6
Light blue cut (band III-a)	95.7
Top 8 mm. (bands IV, V, VI)	42.2
Total	360.1

improve separations and also as a criterion of purity or homogeneity of the various fractions that were eluted.

Mobile Phase.—Since crude fraction D was completely soluble in chloroform, this solvent was chosen as the chief component of the mobile phase. Experiments using chloroform containing varying amounts of butanol gave generally poor results, with few clear-cut separations; results with ethyl acetate were little better. The best results were obtained with chloroform containing a small amount of ethyl alcohol. Mixtures of chloroform containing up to 5% ethyl alcohol did not remove any appreciable amount of the buffered stationary phase. In this work, mixtures of chloroform with 1–3% ethyl alcohol were used routinely.

Measurement of Separations.—Progress of the separations was followed by collecting the eluate in 5-cc. or 10-cc. fractions in graduated cylinders, transferring the fractions to tared aluminum dishes,[4] allowing the solvent to evaporate, and weighing the residues. From these data effluent-concentration curves were determined and the various fractions of eluate combined accordingly. Use of these dishes also permitted the collection of the separated components in a readily recoverable form. The dishes were prepared by numbering; rinsing in hot chloroform, hot water, and hot alcohol, in succession; air-drying, and weighing. With reasonable care their weights did not vary more than ±0.2 mg. over a period of months.

Preparation and Operation of a Typical Column.—All columns were prepared in 1.7 x 60-cm. glass tubes fitted at the top with a 24/25 joint for the application of air pressure. Silicic acid, 15 Gm., was mixed intimately in a mortar with 13 cc. of 0.5 *M* phosphate buffer of *p*H 8.5, and with this hydrated silicic acid was mixed sufficient chloroform, saturated with buffer, to form a thick but fluid paste. The paste was poured into the tube, using a generous amount of chloroform to rinse the mortar. Application of air pressure resulted in compression of the column and removal of excess chloroform. Repeated additions of chloroform with the application of air pressure, keeping a slight "head" of chloroform on the top of the column at all times, resulted in a uniform packing. The excess chloroform was then allowed to drain from the column and a long glass rod, formed into a plunger at one end, used to smooth the top surface. The weight of the glass rod was usually sufficient to form a firm surface. Columns packed in this fashion had a flow rate of approximately 1 cc. per minute and their height was 1 cm./Gm. of silicic acid.

Samples of crude fraction D, dissolved in the minimum amount of chloroform, were introduced directly onto the surface of the column dropwise through an elongated glass funnel whose tip was about 1.5 cm. above the top of the column, and the funnel rinsed with a little chloroform. The average weight of sample used on each column was approximately 300 mg. The use of smaller samples produced more complete separations. In later work, when it was desired to obtain considerable amounts of fraction IV (see below), columns were operated with 700-mg. samples, but in those cases fractions I and II appeared in the eluate as one fraction.

Elution of the column was then carried out, using chloroform containing 1–3% ethyl alcohol, and collecting the eluate in 5-cc. portions. The use of a siphon arrangement was found convenient to keep a constant supply of eluant on the top of the column. Residues obtained by evaporation of the eluate portions were then combined, as nearly as possible according to the separations indicated by plotting eluate fraction number *vs.* mg. per 5-cc. fraction.

With columns operated in this manner it was found that crude fraction D could be separated into five components which were designated fractions I, II, III, IV, and V. Fraction V was composed of the material not eluted from the column but which was stripped, along with the stationary phase, by eluting with methyl alcohol. Figure 3 shows a typical separation obtained in this manner. That

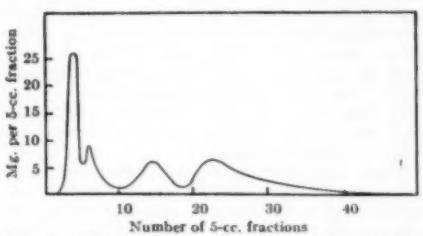

Fig. 3.—The separation of fraction D by partition chromatography.

these results could be duplicated was demonstrated by the fact that the first four fractions were eluted in almost the same portions of eluate when seven columns were run in succession. On the basis of these results the approximate composition of crude fraction D was found to be:

Fraction	I	II	III	IV	V
% of total	12.1	9.35	9.6	20.5	45.0

Isolation of Sabatine from Fraction IV.—Since fraction IV constituted about 20.5% of crude fraction D and appeared to be relatively pure, it was investigated more fully. In order to obtain more of this fraction, partition columns were operated, in the manner described above, in groups of two or three. In these runs, 15 to 17 portions of 5 cc. each

[4] No. 2869 moisture dishes, aluminum, obtained from Schaar and Co., Chicago.

of eluate were collected at the beginning, this volume having been determined, on the basis of previous operations, to be the amount of eluant required for the appearance of fraction IV in the eluate. One large fraction of 150 cc. of eluate was then collected, representing fraction IV.

Fraction IV, obtained in this way, was purified by repeated rechromatography on the partition column, with the typical results shown in Fig. 4. All attempts to crystallize the purified material were unsuccessful until, through no apparent reason, a few needle-like crystals were observed in the residue of one of the aluminum dishes. The residues in the remaining dishes, representing fraction IV in that run, were dissolved in small amounts of chloroform and seeded with these crystals. Upon concentration, all of them solidified to crystalline masses. With the aid of the seed crystals, all samples of the purified fraction IV yielded to ready crystallization from chloroform.

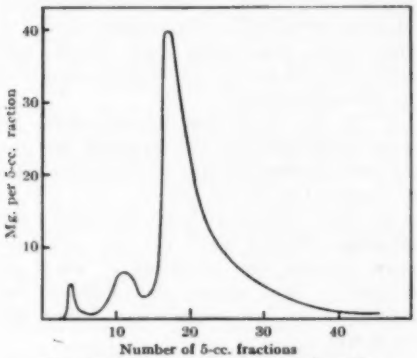

Fig. 4.—The purification of fraction IV.

PROPERTIES OF SABATINE

The white, needle-like crystalline product to which we have given the name "sabatine," when crystallized from chloroform, melts sharply at 210 to 210.5°,[5] $[\alpha]_D^{25°} = -11.3°$ (C = 2.42 in absolute alcohol). In the proportions commonly used for qualitative solubility tests it is soluble in 5% HCl, methanol, ethanol, acetone, dioxane, ethyl acetate, benzene and chloroform, and insoluble in water, Skelly B, ether, and cyclohexane. It dissolves in 5% NaOH upon standing or upon addition of excess NaOH. It gives a positive test for nitrogen, and also a positive test for chlorine. Quantitative chloride determination, using the amperometric titration with silver nitrate solution, shows the presence of chlorine corresponding to 7.60% chloroform. Since the melting point is sharp and the product lost no weight when dried for twenty-four hours at 100°/<0.5 mm., this may be regarded as chloroform of crystallization.

Another sample was crystallized with difficulty from a mixture of cyclohexane and acetone. After drying for twenty-four hours at 110°/<0.5 mm., it melts at 255–257° (dec.) when placed in a cool bath and the temperature gradually raised; when placed in a bath at 220–230°, it melts immediately. The product from cyclohexane-acetone gives a negative test for chlorine.

Several crystalline salts and derivatives of sabatine were prepared, including the hydrochloride, m. p. > 282°; aurichloride, m. p. not constant; diliturate, m. p. 258° (dec.); and sulfate. The alkaloid shows almost no absorption in the ultraviolet region.

Analytical Data.—For product from chloroform, C = 62.27,[6] 62.22; H = 8.25, 8.27; N = 2.27, 2.25 (by titration); sapon. eq. = 624, 618; equiv. wt. = 617.4, 623.7.

For product from cyclohexane-acetone, C = 63.68, 65.01; H = 9.04, 8.82 (different samples); N = 2.51, 2.50 (by titration); equiv. wt. = 556.8, 558.6.

Hydrolysis of Sabatine; Isolation of Sabine and Acetic Acid.—To 2.014 Gm. of sabatine, recrystallized from chloroform and dissolved in 2 cc. of methyl alcohol, was added 15 cc. of approximately 1 *N* methyl alcoholic KOH and the mixture refluxed for two hours. After fifteen minutes there began to separate a white crystalline substance which, upon later investigation, was shown to be potassium chloride, resulting apparently from decomposition of the chloroform of crystallization. The clear hydrolysis mixture was allowed to stand overnight in the refrigerator, the KCl removed by filtration, and the filtrate adjusted to a *p*H of 9.0 by addition of 5% H_2SO_4. The white amorphous precipitate which formed at this point (potassium sulfate) was filtered and the filtrate distilled to remove the methyl alcohol. The tan gummy residue which remained partially crystallized as large salt-like crystals after a few days. Without further purification they melted over a range, 180–189°, and were soluble in methanol, ethanol, chloroform, 5% HCl, acetone, ethyl acetate, and isopropyl ether, but could not be crystallized from any of these solvents.

For purification, this alkanolamine, to which we have given the name "sabine," was converted to the crystalline sulfate, which was recrystallized several times, and then converted back to the free base by extraction from an alkalinized solution with ether. The thus purified amorphous product melted indefinitely over a range from 163–178°, $[\alpha]_D^{25°} = -33.0°$ (C = 4.27 in absolute alcohol).

Anal. C = 64.78; H = 9.61; N = 2.60, 2.65 (by titration); equiv. wt. = 536.8, 529.3.

The aqueous portion of the hydrolysis mixture, remaining after removal of the methyl alcohol and sabine, was distilled. A Duclaux determination on this distillate gave values of 6.2, 6.8, and 7.4, corresponding to acetic acid. The *p*-phenylphenacyl ester, prepared from the distillates remaining from the Duclaux determination, melted at 111–112° and gave no depression in a mixed melting point with the same ester of a known sample of acetic acid.

Steam distillation of the aqueous portion, remaining after the removal of acetic acid, followed by continuous extraction with ether, showed the presence of no other acids in the hydrolysis mixture.

[5] Melting points obtained by Anschutz thermometers.

[6] We are indebted to Professor A. L. Wilds, Chemistry Department, University of Wisconsin, for combustion data.

PHARMACOLOGICAL TESTS ON SABATINE[7]

The effects of sabatine on the heart, blood pressure, respiration and intestinal motility were studied in dogs anesthetized with phenobarbital sodium. Intravenous doses of 0.1 mg./Kg. by vein produced a fall in blood pressure of 10 mm. Hg which returned to normal almost immediately. When the dose was increased to 0.4–1.0 mg./Kg., the drop in blood pressure was more pronounced and lasted for fifteen to thirty minutes following 1.0 mg./Kg. This dose also decreased the heart rate from 140 to 120 per minute, and produced a negative T wave on the electrocardiogram. The blood pressure and electrocardiogram appeared normal fifteen minutes after the injection.

Small doses did not affect respiration or intestinal motility. Larger doses, up to 1.0 mg./Kg., increased the respiratory rate. It required 1.0 mg./Kg. to produce any change in intestinal motility. This dose decreased the motility but did not abolish the segmental movements.

The acute toxicity was determined in albino mice by intravenous injection. The median lethal dose (LD_{50}) was 8.95 ± 0.37 mg./Kg.

DISCUSSION AND CONCLUSIONS

On the basis of quantitative chloride determinations, sabatine appears to crystallize from chloroform with $^2/_5$ mole of solvent of crystallization. This factor, plus the difficulty in crystallizing it from other solvents, has complicated the work on its characterization. That sabatine contains only one nitrogen is indicated, in part, by the single change in the potentiometric titration curve, as well as by the equivalent weight and saponification equivalent. Analytical data obtained on the sample from chloroform, when corrected for the 7.60% $CHCl_3$, are: C = 66.85; H = 8.85; equiv. wt. = 570.5, 576.3; sapon. eq. = 571, 577, respectively, as compared with similar data for the sample from cyclohexane-acetone: C = 63.68, 65.01; H = 9.04, 8.82; equiv. wt. = 556.8, 558.6. That sabatine is probably a monoacetate is indicated by the difference in its equivalent weight from that of the hydrolysis product, sabine.

Based on the analytical data obtained, sabine would have a molecular formula approximating $C_{28}H_{51}O_7N$, mol. wt. = 526, while sabatine, if considered its monoacetate, would have the formula $C_{31}H_{53}O_8N$, mol. wt. = 567. While the equivalent weight and saponification equivalent data agree fairly well for these formulas, and the combustion data agree also in the case of sabine, the combustion data in the case of sabatine is not in agreement. It is thus not possible at this time to assign a definite molecular formula to either the ester alkaloid, sabatine, or its hydrolysis product, sabine. Further work is in progress on their characterization.

[7] We are indebted to Dr. K. K. Chen of the Lilly Research Laboratories for these tests.

SUMMARY

1. The application of adsorption chromatography to the water-soluble fraction of alkaloids obtained from sabadilla did not produce any reasonably clear-cut separations. The use of ultraviolet light was of some assistance in following the development of the chromatogram, but such a practice might lead to erroneous conclusions, since one portion of the column, which showed no fluorescence, was found, upon elution with methyl alcohol, to have adsorbed 17 per cent of the alkaloidal mixture.

2. By use of partition chromatography the water-soluble fraction was separated into five different components which have been designated as fractions I, II, III, IV, and V.

3. From fraction IV there was isolated a new crystalline alkaloid which has been given the name sabatine, the properties of which are described. Upon alkaline hydrolysis it was cleaved to the new alkanolamine, sabine, and acetic acid.

4. Preliminary pharmacological tests on sabatine are described.

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Behavior of Some Inorganic Anions as Bases in Glacial Acetic Acid*

By TAKERU HIGUCHI and JESUSA CONCHA†

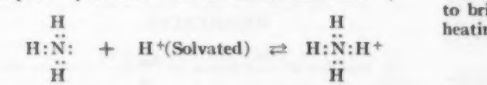
Data are presented to show that all common inorganic anions, except bisulfate, act as bases in glacial acetic acid. Supporting evidence includes results obtained by titrating a number of alkaloidal salts and many inorganic salts. Theoretical basis for this behavior is discussed.

A NUMBER of papers have recently appeared reporting advantages of titrating weak bases in glacial acetic acid and other nonaqueous solvents as compared to their determination in aqueous media (1–4). Aniline, for example, can be titrated readily in glacial acetic acid with perchloric acid, whereas in water such titration leads to indefinite end points. It is evident that the method holds a great deal of promise in its application to pharmaceutical analysis. Users of the method, nevertheless, should exercise some caution in interpreting results obtained from analysis of samples containing salts.

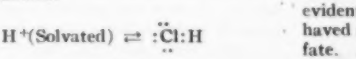
As pointed out in another publication (5), protonated acetic acid is such a strong acid that even such a substance as chloride ion, Cl^-, behaves as a base relative to it It was shown that chlorides and procaine hydrochloride are titrated as bases in glacial acetic acid with perchloric acid, if commonly used indicators are employed. In the present communication, the results of titration in glacial acetic acid of a number of additional anionic types which are normally considered to be nonbasic are presented.

THEORY

According to the modern concept of acids and bases, a base is any substance which will accept a proton. Thus, ammonia is a base because it can acquire a proton to form the ammonium ion:



It has been pointed out that a chloride ion acts as a base in a similar fashion:



The proton affinity of the chloride ion is, of course, much weaker than that of ammonia, a relatively strong proton donor being necessary to form hydrogen chloride. In glacial acetic acid the thermodynamic activity of the latter compound at any given concentration is much higher than in water, being readily volatilized from solution by boiling. This behavior can be used to force the above reaction completely to the right.

Other anions such as bromide, nitrate, nitrite, sulfate, phosphate, monohydrogen phosphate, dihydrogen phosphate, formate, etc., also behave as bases to varying extents in glacial acetic acid. Protonated acetic acid which is formed through reaction of perchloric acid with the solvent is a stronger acid than the conjugate acids of these bases, thus acting effectively as proton donor in each case. It is apparent that if an indicator, whose pK_I value lies approximately midway between the pK_a values of the protonated acetic acid and the conjugate acids, were chosen, it would be possible to titrate these bases in glacial acetic acid.

In the present investigation, a study has been made of several anions which may behave as bases in glacial acetic acid with different indicators. Some earlier results are included for comparison purposes. No attempt was made in this study to obtain extremely accurate quantitative results, but rather to obtain information as to how these substances may influence analytical results.

EXPERIMENTAL

The following procedure was employed in obtaining the data reported:

The weighed sample (0.1 to 0.5 Gm.) was placed in a 250-ml. Erlenmeyer flask with 50 ml. of glacial acetic acid. The mixture was brought to a boil to facilitate solution of the salt, and a few drops of an indicator (0.1% in benzene or glacial acetic acid) were added. The solution was then titrated with a standardized perchloric acid-acetic acid solution (0.04 to 0.2 *M*).

With the chlorides, the solution was reheated near the end point to sharpen the indicator change. Where relatively insoluble salts, e. g., nearly all of the inorganic salts, were analyzed, it was necessary to bring them gradually into solution by repeated heating and titration.

RESULTS AND DISCUSSION

The results of acidimetric titration of several common inorganic salts are given in Table I. It is evident from the data that all the anions tested behaved as monoacidic bases except sulfate and bisulfate. A partial explanation for the latter behavior lies in the fact that sulfuric acid is a relatively strong acid, second only to perchloric acid among the common acids. Thus, in the reaction

$$SO_4^= + H^+(\text{Acetic Acid}) \rightleftarrows HSO_4^-$$

the equilibrium lies far to the right. However, in the succeeding reaction,

* Received November 20, 1950, from the Research Laboratories, School of Pharmacy, University of Wisconsin, Madison.

† Fellow of the University of the Philippines.

TABLE I.—ACIDIMETRIC TITRATION OF SEVERAL COMMON INORGANIC SALTS

Anion Type	Compound	Indicator Used	Gm. Equiv. of Acid/ Mole of Sample
Chlorides	Sodium chloride	α-Naphthol-benzein	0.997
	Potassium chloride	α-Naphthol-benzein	0.991
	Ammonium chloride	α-Naphthol-benzein	0.999
	Calcium chloride	Methyl violet	1.907
Bromide	Potassium bromide	α-Naphthol-benzein	0.976
Nitrate	Ammonium nitrate	Methyl violet	0.962
Nitrite	Sodium nitrite	Methyl violet	1.003
Sulfate	Potassium sulfate	α-Naphthol-benzein	1.26
Bisulfate	Sodium bisulfate	Methyl violet	0.16

TABLE II.—ACIDIMETRIC TITRATION OF SEVERAL SALTS OF ORGANIC COMPOUNDS

Anion Type	Compound	Indicator Used	Gm. Equiv. of Acid/ Mole of Sample
Chlorides	Procaine hydrochloride	α-Naphthol-benzein	1.94
	Procaine hydrochloride	Quinaldine red	0.97
	Pilocarpine hydrochloride	Methyl violet	1.00
	Histidine dihydrochloride	Methyl violet	2.04
	Glutamic acid hydrochloride	Methyl violet	0.99
Nitrate	Pilocarpine nitrate	α-Naphthol-benzein	0.95
Sulfate	Quinine sulfate	α-Naphthol-benzein	3.00
	Quinine sulfate	Quinaldine red	2.87
Phosphate	Codeine phosphate	Quinaldine red	0.996
	Codeine phosphate	Methyl violet	0.997
	Histamine phosphate	Quinaldine red	2.05
	Histamine phosphate	Methyl violet	2.03

$$HSO_4^- + H^+(\text{Acetic Acid}) \rightleftarrows H_2SO_4$$

the reaction is far from complete. Considerable difficulty was encountered in titration of sulfate and bisulfate because of their poor solubility. Titration of nitrates was complicated by the oxidizing tendency of the nitric acid formed.

The results of titration of organic compounds containing inorganic anions are listed in Table II. The titration of these substances was facilitated by their higher solubility in glacial acetic acid.

Procaine hydrochloride contains two basic groups, the chloride ion and the *p*-amino group. The latter may remain as such or may react with glacial acetic acid to yield the base, acetate. In either case, whether present as an acetate ion or as a free amino group, the basicity resulting from the original *p*-amino group is considerably stronger than that arising from the chloride ion. By using suitable indicators, therefore, it is possible to differentiate between the two contributions. Thus, with quinaldine red, which changes color in the relatively weakly acidic region, procaine hydrochloride takes up one equivalent of acid per mole. With α-naphtholbenzein, which changes color on the strongly acidic side, two equivalents are taken up.

The acid consumptions of the three remaining chlorides in this group are what is to be expected on the basis of their chloride content. The same is true of pilocarpine nitrate.

The three-equivalence-per-mole value for quinine sulfate is exactly what would be expected if we assume that sulfates are titrated only to bisulfates as previously discussed. Thus, the over-all equation would be

$$(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4 + 3H^+(\text{Acetic Acid}) \rightarrow 2(C_{20}H_{24}N_2O_2 \cdot 2H^+) + HSO_4^-$$

The two phosphates analyzed were both titrated to phosphoric acid, even with quinaldine red as the indicator. This is to be expected since phosphoric acid is a relatively weak acid ($pK_a = 2$).

From the data presented and above discussion it is evident that very few salts, both inorganic and organic, are neutral in glacial acetic acid. This fact should be kept in mind when weak bases are determined in this solvent. For example, the quinine content of a mixture of quinine and quinine sulfate cannot be directly determined by the method since sulfate ions will also take up the acid.

The behavior of the salts in this manner, however, may have some value in analysis of the salts themselves.

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Note

A Note on the Depyrogenization of Infusion Solutions by Hydrogen Peroxide*,†

By E. MENCZEL‡

THE hydrogen-peroxide depyrogenization method of Taub and Hart (1) for the preparation of sterile pyrogenic-free solutions of saline and saline-dextrose for intravenous infusions was introduced at the Government Hospital "Yarkon," Tel-Aviv, in August, 1949. The distilled water in use at that hospital is obtained from a commercially operated still, and solutions prepared from it proved to be highly pyrogenic. The detoxification of pyrogens according to Taub and Hart's method was applied. Through the preparation of 4,000 L. of saline and saline-dextrose by this method, slightly modified and strictly controlled, no pyrogenic reaction occurred. Bearing in mind that any new procedure in pharmaceutical practice may be liable to unforeseen interferences, we subjected the reagents and solutions prepared to a variety of control tests, and it was found that part of these controls are highly essential for the achievement of proper results.

One modification of the Taub and Hart method involved replacing the Soxhlet apparatus with ordinary large Pyrex boiling flasks. This was done to facilitate the preparation of large quantities of solutions. These boiling flasks were stoppered with ordinary cotton gauze stoppers which did not interfere with smooth boiling. On the completion of the boiling, the solution was adjusted to its original volume by adding a quantity of similarly depyrogenized distilled water equal to the volume evaporated. This simplification did not reduce the accuracy of the method and its validity compared to the original one.

The Taub and Hart method utilizes extraneous reagents, and every precaution should be taken to insure that the infusion solutions are free from even traces of these reagents. Notwithstanding the fact that Taub and Hart indicate that their method eliminated any residual peroxide (tested by titration with standard permanganate), and that the depyrogenized injection water prepared by their method passed the U. S. P. permanganate test for limit of oxidizable substances, our saline solutions similarly depyrogenized failed to meet either test. As both tests utilize permanganate, and no possible differentiation could be expected between residual hydrogen peroxide and oxidizable substances, it was thought advisable to test for the presence of residual hydrogen peroxide by a method different from titration with permanganate before the U. S. P. limit test for oxidizable substances was applied.

There are a large number of tests for hydrogen peroxide in the literature, most of which, including those of the British Pharmacopeia 1948 (2) and the United States Pharmacopeia XII (3) tests, were not sensitive at the concentration used. Fair results, however, were obtained with Feigl's (4) spot test with ferri-ferric cyanide reagent, when modified to obtain greater sensitivity. The reagent, a mixture of equal quantities of 0.4% ferric chloride solution and 0.8% potassium ferricyanide solution, is sensitive to 0.08 μg. of hydrogen peroxide in a drop. The sensitivity of the test, however, may be increased twofold by the following modification. One hundred cubic centimeters of solution to be tested, to which ten drops of this reagent is added, acquires a blue color or tint if traces of hydrogen peroxide are present. Dextrose does not interfere with this reaction in spite of its reducing properties, but the color should be examined within ten minutes. It should be noted that ferri-ferric cyanidt reagent, mixed with freshly double-distilled water, develops a blue color after several hours of standing.

Further tests indicated that ferri-ferric cyanide reacted immediately with distilled water of questionable value to which no hydrogen peroxide had been added, as well as with many solutions which proved to be pyrogenic. A solution of one-fourth per cent typhoid vaccine (usual standard) in double-distilled water, and a similar solution of cholera vaccine, reacted with ferri-ferric cyanide reagent and failed in the same manner to pass the U. S. P. permanganate limit test of oxidizable substances. As both tests are based on oxidation mechanisms, no differentiation between traces of hydrogen peroxide and oxidizable substances could be established by the application of either. Further tests indicated that ferri-ferric cyanide is a sensitive reagent for the detection of both oxidizable substances and pyrogenic contamination in very minute quantities. Additional study is still necessary to confirm these preliminary tests which indicate that this reagent may serve as a chemical test for pyrogens, and may even replace the U. S. P. permanganate test for limit of oxidizable substances. An advantage of such a replacement is that it could be applied to dextrose solutions intended for injection intravenously. Infusion solutions depyrogenized by the Taub and Hart method should be tested for traces of hydrogen peroxide, by a reagent which is not reduced by oxidizable substances, before any limit test of oxidizable substances is attempted.

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†Submitted for publication with the approval of the Medical Superintendent, Government Haospital, Tel-Aviv.
‡ The author is indebted to Dr. Rapaport for the assistance given in carrying out this work.

Hydrogen peroxide serves as sensitive reagent for tetravalent titanium according to Feigl (5), Strebinger (6), Treadwell (7), and Flagg and Line (8). By reaction between tetravalent titanium and hydrogen peroxide, in the presence of sulfuric acid, a yellow-red color is obtained which varies according to concentration. The authors cited do not suggest that this reaction is a sensitive test for hydrogen peroxide. Rapaport (9, 10), however, used this reaction to trace the residual hydrogen peroxide in the determination of iron in whole blood, and according to his personal advice, the same reaction was applied to test the presence of residual hydrogen peroxide in solutions depyrogenized by the Taub and Hart method. The reagent used was prepared by dissolving 0.5% titanium dioxide in boiling concentrated sulfuric acid containing 4% ammonium sulfate. By a dilution method, it was found that this reaction is sensitive to 0.0002% hydrogen peroxide when the test is carried out by adding 20 drops of the reagent to 100 cc. of solution to be tested. Owing to the light shades of yellow color, blank tests are advisable. The effect of oxidizable substances was studied by adding dextrose solutions of different concentrations to this reagent, but no interferences were encountered, even after a week of standing. A one-fourth per cent solution of typhoid vaccine (usual standard) did not affect the reagent whatsoever. On the other hand, positive tests were obtained with saline solutions prepared according to the Taub and Hart method. Thus it was established that a definite residual amount of peroxide is still present in such solutions and that this interference should not be neglected. However, by boiling the same solution with 0.1% manganese dioxide for a quarter of an hour, at least, the test proved to be negative; furthermore, these solutions passed the U. S. P. and British Pharmacopeia 1948 limit test for oxidizable substances as well as the ferri-ferric cyanide test. The Taub and Hart method should therefore be modified by additional boiling of saline solutions for a quarter of an hour with 0.1% manganese dioxide in order to eliminate any detectable hydrogen peroxide. The addition of manganese dioxide to boiling solutions produces excessive foaming which may subsequently cause loss of solution. This can be avoided by cooling the solution to 70° after it has been boiled for one hour with hydrogen peroxide, adding the manganese dioxide and resuming the boiling for a quarter of an hour. The same holds true for saline-dextrose solutions, except that 1% activated charcoal is used instead of manganese dioxide.

The other extraneous reagent used in the Taub and Hart method is manganese dioxide from which, according to Broughton and his co-workers' (11), catalytic studies on the destruction of hydrogen peroxide, no solution of manganese ion is to be expected. Our control tests confirmed that no residual manganese ion could have been traced by Feigl's benzidine spot method, even when modified to increase its sensitivity by applying it to 50 cc. of solution. On the other hand, saline-dextrose solutions dissolved considerable traces of manganese ions, and oxidizable substances were produced which proved to be pyrogenic. It should be noted that in no instance should manganese dioxide be used during the preparation of saline-dextrose solutions intended for intravenous injection.

SUMMARY

The validity of the Taub and Hart method for depyrogenizing infusion solutions has been confirmed practically. Control tests for residual hydrogen peroxide and oxidizable substances were studied, and preliminary tests indicated that ferri-ferric cyanide reagent is sensitive to oxidizable contamination in infusion solutions which are pyrogenic. In order to eliminate any detectable residual hydrogen peroxide, it was concluded that additional boiling for a quarter of an hour with manganese dioxide in saline solutions and activated charcoal in saline-dextrose solutions is highly essential.

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Book Reviews

Colorimetric Determination of Traces of Metals. 2nd ed. By E. B. SANDELL. Interscience Publishers, Inc., New York, 1950. xix + 673 pp. 15 x 23.5 cm. Price $9.

This edition has been considerably expanded over the first one. It has the same general organization, being divided into a general part on Colorimetric Trace Analyses and a special section on Procedures for the Determination of Specific Metals. The first portion discusses the general concepts of what constitutes a trace metal and a trace analysis, and presents general methods for isolation of the material to be analyzed. Included is a presentation of colorimetric and spectrophotometric methods in general, with considerable attention devoted to the various reagents that are used for detection of trace elements.

In the second section each metal is taken up separately and the analytical methods that have been successful for the given metals are presented. There are detailed directions for the assay, along with some criticism and evaluation of the procedure. References to the original literature describing the development of each method and its application to various substances are given.

The completeness with which the book presents its subject, and the care with which it was written, make it of outstanding value to the analytical chemist.

Methods in Medical Research. Vol. 3. RALPH W. GERARD, Editor-in-Chief. Year Book Publishers, Inc., Chicago, 1950. xii + 312 pp. Illustrated. 14 x 22.5 cm. Price $7.

This volume represents a continuation of the original idea of presenting in considerable detail specific techniques that are used in medical research. Each method and procedure is written by someone with considerable experience in the field, and it is usually found that the procedures have not been published in detail elsewhere except as numerous scattered articles.

The subject matter of this volume is divided into four sections. The first section, Genetics of Micro-Organisms, contains procedures for isolation and characterization of biochemical mutants of bacteria, yeast, and neurospora. Here one finds details as to suitable culture media, transfer techniques, and apparatus.

Section II, Assay of Neurohumors, presents methods for estimating the activity of cholinesterases, choline acetylase, and methods used for the determination of adrenaline and nor-adrenaline, also methods for evaluating effects of inhibitors.

Section III, Selected Psychomotor Measurement Methods, discusses procedures for making physical work and strength tests, reaction time and coordination studies, dexterity and eye-movement coordination measurements.

Section IV, Methods for Study of Peptide Structure, discusses the microbiologic methods for assay of amino acids, use of 1:2:4-fluorodinitrobenzene, in identifying peptide constituents, and the use of synthetic methods in the study of peptides.

These volumes of *Methods in Medical Research* are particularly important to the laboratory workers, and in many instances they will save considerable time by obviating much library research.

Jan Ingenhousz, Plant Physiologist (With a History of the Discovery of Photosynthesis). By HOWARD S. REED. Reprinted from the Chronica Botanica, Vol. 11, No. 5/6, pp. 285–396. The Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York City, 1950. Illustrated. 17.5 x 26 cm. Price $3.

This account, representing a reprint from No. 5/6 of Volume 11 of the *Chronica Botanica*, is another of the meritorious publications of general interest to the history of science which owe their appearance to the initiative of Dr. Frans Verdoorn, the editor of the *Chronica Botanica*, and which have done so much to preserve the memory of the great and to make it fruitful for present understanding and future research.

Not that Ingenhousz and his pioneer work in photosynthesis ever have been forgotten or underestimated by those interested in the history of the natural sciences. Although one looks almost in vain for his name in the general encyclopedias, the fact and the meaning of the Dutch physician's discovery of the action of light on the green cells of plants has been given full credit in the scientific literature concerned, and in the respective historical accounts. Hermann Kopp, in his fundamental *Geschichte der Chemie* (Braunschweig, 1843–1847) refers five times to Ingenhousz, and Ferchl-Süssenguth, in their *Kurzgeschichte der Chemie* (Mittenwald, 1936)[1], call the photosynthesis of green plants "the most important of all chemical processes" and attribute to its discoverer "immortality."

The classical work in which Ingenhousz reported on his experiments and made known his conclusions, however, has been out of print and is, as Reed puts it, "generally known only through second-hand quotation."

Here, then, the text of the English original of 1779, with a few interpolations from the French edition of 1787, is reproduced. Still more, it has been annotated by Reed in such a masterly way that even the less informed reader cannot miss the significance of the individual statements made and the general conclusions drawn by Ingenhousz. In addition, Reed's comments offer an interesting picture of the scientific situation of this period. His explanation of the discrepancies between the ideas of

[1] The book was translated into English under the title "A Pictorial History of Chemistry" (London, 1938). Strangely enough, the reference to the "immortality" achieved by Ingenhousz is missing in the English version.

Scheele and Priestley, both based on partial views only, is quite as illuminating as his interpretation of the controversy between Ingenhousz and other contemporary scientists, especially Rumford and Sennebier.

The book is not only enlivened, but substantially enriched by a number of rather rare and well-chosen illustrations. Readers in this country will notice with special interest two letters written to Ingenhousz by Benjamin Franklin.—GEORGE URDANG.

Pharmaceutical Emulsions and Emulsifying Agents. By LAWRENCE M. SPALTON. Chemical Publishing Co., Inc., Brooklyn, N. Y., 1950. vii + 132 pp. Illustrated. 14 x 22.5 cm. Price $3.75.

This is a book intended to introduce the pharmacist or student to the preparation of emulsions and to the use of some of the newer types of emulsifying and suspending agents.

The text explains simply and briefly how emulsions are formed, and what constitutes an emulsion. It describes the various common types of emulsifying agents and attempts to point out the usefulness of each type. Agents that are used for improving the stability of emulsions are included, and the preservatives that are of value are discussed.

A large number of formulas for emulsions and emulsifying ointment bases are included as illustrations of various principles and methods. Finally, there is a classified list of emulsifying agents, and the American reader will be pleased to find that U. S. trade names are included for most of the emulsifying agents.

Each agent in the table is classified according to type, and reference is made to the discussion of this agent in the text.

Although this book is not as detailed as would be desired by the research worker, it constitutes an excellent introduction to the subject of emulsions and to the use of some of the newer emulsifying agents. It should be quite useful to the practical pharmacist, the student, and to the small-scale manufacturer.

The Enzymes—Chemistry and Mechanism of Action. Vol. I, Part I. Edited by JAMES B. SUMNER and KARL MYRBÄCK. Academic Press, Inc., New York, 1950. xvii + 724 pp. Illustrated. 15.5 x 23.5 cm. Price $13.50.

This is the first of a set of books planned to survey the field of enzymology, and this series is most welcome, indeed, to all biochemists, pharmacologists, and workers in related fields. The expansion of knowledge in the field of enzymology has been so great in recent years that concepts of a few years ago have been, in many instances, completely replaced by knowledge based on more exact measurements and more careful research. The literature on enzymes has been so voluminous and individual reports so specialized, that a survey such as this will be extremely valuable.

The chapters are written by authoritative workers in the various fields. The book includes an excellent introduction which reviews the physical nature and properties of enzymes and co-enzymes, along with some introductory material on kinetics.

There is an excellent chapter on kinetics and physical chemistry of enzymes which includes mathematical treatment of enzyme reactions and the factors affecting reactions. Descriptions are given of the methods of measurement of molecular weight, use of electrophoresis in determination of enzyme purity, etc. It seems that this particular section might have deserved even more detailed treatment and the use of more examples from the literature, although as it now stands it comprises almost 50 pages.

Other subjects included in this book are Enzyme Specificity, Enzymes in Relation to Genes, Viruses, Hormones, Vitamins and Chemotherapeutic Drug Action, Cytochemical Foundations of Enzyme Chemistry, Modern Aspects of Enzymatic Adaptation, Enzymes Hydrolyzing Fats and Esters, Acetylcholine Esterase and Cholinesterase, Phosphatases, Metaphosphate and Its Enzymatic Breakdown, Sulfatases, Invertase, α-D-Glucosidases, β-Glucosidase, Hydrolysis of Galactosides, Mannosides, and Thioglycosides, β-Glucuronidase, and Amylases and the Hydrolysis of Starch and Glycogen.

One of the sections that is particularly well treated is that of enzyme inhibition, and this subject is of special interest to those engaged in medicinal chemistry.

This book will be an important and essential reference to all who work in the field of enzyme chemistry and for many in the field of medicinal chemistry.

Organic Reagents for Organic Analysis. 2nd ed. By the Staff of Hopkin and Williams Research Laboratory. Chemical Publishing Co., Inc., Brooklyn, N. Y., 1950. 263 pp. 14 x 22 cm. Price $5.

This is a simple but very useful book for the organic chemist. It lists groups of selected reagents, and other reagents that are suitable for the preparation of derivatives of various organic compounds. In the second section, it treats each of the selected reagents in more detail, describing the reagents and their properties, and presenting in brief form the directions for using the reagents. Of special value is the inclusion of references for each procedure.

This type of organization makes the book very practical in the laboratory. If one wishes to identify a secondary amine, for example, he can turn to the section on amines, secondary, and find a list of about 12 selected reagents useful in preparing derivatives. He can then go back to the tables of derivatives which list most of the secondary amines and, in a moment, can select which derivative will give conclusive identification. Having decided upon the reagent, he can turn to the section of the book where the use of that reagent is described and obtain directions for carrying out the reaction and isolating the product.

It seems that the availability of the list of selected reagents, which are stated to have been selected with great care, will be a special convenience to the chemist, and will, perhaps, avoid many unsuccessful trials in preparing derivatives for characterization.

For teaching purposes, this book might conceivably be used as the main text, but it seems more suitable as an auxiliary reference. By all means, it seems that the student should become familiar with it during a course in qualitative organic chemistry.

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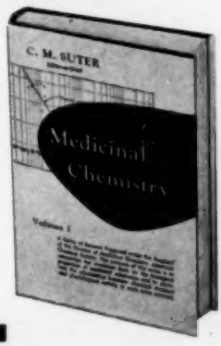

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